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Award Number: W81XWH-11-2-0018

TITLE: Prevention of Trauma/Hemorrhagic Shock-Induced Mortality, Apoptosis, Inflammation and Mitochondrial Dysfunction Using IL-6 as a Resuscitation Adjuvant

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

These studies will determine if IL-6 merits consideration as a resuscitation adjuvant for use by medics at the time of Hextend administration to soldiers suffering from severe battlefield injuries. We demonstrated that leukocyte apoptosis is not sufficiently robust to use as a biomarker to guide patient selection for IL-6. However, we demonstrated that kidney injury accompanies heart, lung, liver and leukocyte apoptosis in rat T/HS and occurs within 4 hours of resuscitation. Studies are underway to determine if kidney injury biomarkers measured in the urine may be used to guide patient selection and to assess benefit. We identified two proteostasis modulators, Hsp70 and Hsp40, as potential key modulators of liver and lung apoptosis in T/HS that may mediate the beneficial effects of IL-6, which raises the possibility of using clinically available proteostasis modulators to prevent cell apoptosis, if IL-6 proves problematic. Finally, we demonstrated in patients with hemorrhagic shock that increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

15. SUBJECT TERMS
IL-6 as a resuscitation adjuvant for severe battlefield injuries. Kidney injury biomarkers in the urine to guide patient selection and to assess benefit. Proteostasis modulators, Hsp70 and Hsp40, may mediate the beneficial effects of IL-6. Association of increased peripheral blood neutrophil apoptosis with reduced risk of subsequent infection.

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INTRODUCTION:

Trauma complicated by hemorrhagic shock (T/HS) on the battlefield is distinct from the civilian arena especially with regards to clinical diagnosis and resuscitation protocols. Progress towards development of new first-responder resuscitation adjuvants for polytrauma and blast injuries that will maintain tissue viability requires that an agent that demonstrates efficacy in animal models that mimic T-HS and resuscitation in the civilian setting also work in animal models of T-HS that mimic combat casualties and battlefield management. We developed a rat T-HS model in which we demonstrated: 1) 72% mortality at 48 hr, 2) hypovolemic circulatory collapse, 3) left ventricular contractile dysfunction, 4) apoptosis of cardiomyocytes, alveolar epithelial cells, hepatocytes and leukocytes, 5) organ inflammation, 6) organ-specific alterations in the apoptosis transcriptome and 7) increased susceptibility to bacterial infections. Especially notable was the finding that apoptosis and inflammation required resuscitation. Remarkably, use of IL-6 (10 μg/kg) as a resuscitation adjuvant: 1) reduced mortality 5 fold, 2) prevented hypovolemic circulatory collapse, 3) prevented ventricular contractile dysfunction, 4) prevented apoptosis of cardiomyocytes, alveolar epithelial cells, hepatocytes and leukocytes, 5) reduced organ inflammation, 6) normalized the apoptosis and inflammation transcriptome in the heart, lung and liver and 7) reduced T-HS-mediated increased susceptibility to bacterial infections. Importantly from a mechanistic standpoint, results using a pharmacological inhibitor of Stat3 and mice deficient in Stat3β, a dominant-negative isoform of Stat3, demonstrated that virtually all of the beneficial effects of IL-6 were mediated through Stat3 especially Stat3α, which, in addition to its transcriptional role in the nucleus, recently has been demonstrated to support oxidative phosphorylation within mitochondria. Based on these findings, we hypothesize that IL-6 administration at the start of resuscitation will be beneficial to rats and swine subjected to polytrauma and HS that more closely mimic battlefield injuries and resuscitation protocols and that IL-6 merits consideration as a resuscitation adjuvant for use by medics at the time of Hextend administration to soldiers suffering from T-HS.

We have outlined five highly focused Specific Aims to examine this hypothesis:

Aims 1, 2, and 3. To determine the effects of IL-6 administration at the start of resuscitation on survival, vital organ apoptosis, injury, inflammation and mitochondrial dysfunction in two rodent models and one swine model that mimic combat casualties and current battlefield fluid resuscitation strategies (Hextend infusion, 14.3 ml/kg):
  1) Rats subjected to laparotomy and controlled HS (AIM 1),
  2) Rats subjected to femur fracture and T-HS (AIM 2) and
  3) Swine subjected to laparotomy, splenectomy, tissue injury and controlled HS (AIM 3).

Aim 4. To determine the effects of IL-6 administration on the transcriptome induced by these T-HS models in rats and swine.

Aim 5. To determine if leukocyte apoptosis can serve as a biomarker of vital organ apoptosis and injury in T-HS patients.

These studies will establish whether or not the benefits of IL-6 administration will extend to battlefield resuscitation protocols for controlled HS in the setting of moderate to severe trauma and will establish the foundation for clinical trials of IL-6 in civilian injuries involving trauma and HS that mimic severe combat injuries.

BODY:

In our original Statement of Work document, we delineated that Tasks in Aims 1 and 5 would be performed in Year 1 as summarized in the Gantt chart below:

TIMELINE (GANTT CHART)

	YEAR 1	YEAR 2	YEAR 3
Specific Aim 1. Effects of IL-6 in rat Lap/HS model			
	Tasks 1A, B, C and D		
Specific Aim 5. Leukocytes as a marker of organ apoptosis in T-HS patients			
	Tasks 5A, B, C and D		

These Tasks and the progress we have made in each are outlined below.

Specific Aim 1. Determine the effects of IL-6 on survival, organ apoptosis, injury and inflammation and mitochondrial dysfunction in rats subjected to Lap/HS model (timeframe: 18 months)

Task 1A. Optimize "shock load" for Lap/HS model.

Subtask IA1. Amend animal approval to include swine (timeframe=1 month)

This was postponed.

Subtask IA2. Determine survival rate of rats subjected to Lap/HS protocol with shock load=60 min (or appropriate; 10 rats; timeframe 1 month).

In earlier studies performed as part of preliminary studies for this award, we determined that the "shock" load necessary to achieve the target mortality of 50% in the first of our proposed new rat T/HS protocols was 60 min at 35 mm Hg. This protocol includes laparotomy followed by fixed pressure hemorrhagic shock (Rat Lap/HS model). In repeat experiments, we started with a 15-minute period to achieve target MAP of 35 mm Hg followed by 60 min at target MAP. Since there were no deaths, we proceeded to increase the duration of time at target MAP to 75 min, then 90, 120 150 and finally 180 min. It was not until using a "shock" load of 180 min that 50% mortality at 48 hr was observed in a total of 6 rats examined. Thus, we have identified a shock load that will allow us to assess if use of IL-6 as a resuscitation adjuvant provides a survival benefit, as outlined below.

While optimizing the "shock load" for the Rat Lap/HS model, we completed rat studies for a critical experiment at the request of our industry partner (Novartis). Novartis is convinced that prevention of kidney injury is the most compelling pathway for entry into clinical use of human recombinant IL-6 as a resuscitation adjuvant. They have developed a panel of sensitive urine analyte assays to test for kidney injury, which they wanted to use to establish if kidney injury occurred in our standard rat T/HS model and to determine whether kidney injury could be prevented by use of IL-6 as a resuscitation adjuvant. In this experiment, we subjected 6 rats each to either our sham protocol or our standard T/HS protocol, as described (1-3) modified per this grant proposal. Specifically, rats were subjected to trauma (groin incision and bilateral superficial femoral artery cannulation) followed by hemorrhagic shock. Instead of being resuscitated with heparinized shed blood and lactated Ringer's solution, they were resuscitated with Hextend as described in this proposal combined with IL-6 as a resuscitation adjuvant at four doses (0, 3, 10 or 30 mg/kg). Serum was harvested at 24 and 48 hrs; urine was harvested at 4, 12, 18, 24, 36, 42 and 48 hr; and kidneys were harvested at 48. Samples of serum and urine were

sent to Novartis on February 23 to be examined in their kidney injury panel. These studies were intended to answer several important questions: 1) Does kidney injury accompany heart, lung and liver injury in rat T/HS? 2) What is the most sensitive and robust urine test to monitor for kidney injury in this setting? 3) Can IL-6 prevent T/HS-induced kidney injury? 4) If so, what is the optimum dose of IL-6 to use in the rat T/HS studies outlined in our proposal?

We received the results from Novartis on August 2, 2011 (**Appendix 1**). Of the 10 urine markers of kidney injury, 7 demonstrated evidence of substantial increase including GSTYb, NGAL, Kim-1, Cystatin, beta2M, albumin, and clusterin, which indicated that the kidney demonstrated clear evidence of injury in this model of T/HS. Evidence of injury was maximal by 4 hours. The most sensitive and robust urine marker of kidney injury was Kim-1, but most of the 7 indices were nearly equally sensitive. Somewhat to our surprise, however, while there was some suggestion of protection by IL-6 against renal injury determined by Kim-1 and albumin levels at an IL-6 dose of 10 ug/kg, these results were not statistically significant. Neither was there evidence of benefit of IL-6 on liver and heart injury. The inability to demonstrate a clear benefit of IL-6 at any of the three doses was at odds with all of our earlier publications (1-5). Two major hypotheses for this disparity are: 1) the ability of Hextend to adsorb either IL-6 or sIL-6R, and 2) the absence within the Hextend of a factor(s) present within the returned shed blood (previously used for resuscitation) that are necessary for the beneficial effect of IL-6. The leading candidate for this "missing factor" is sIL-6R.

To explore the hypothesis that Hextend adsorbs either IL-6 or sIL-6R thereby blunting its effects, we examined whether Hextend interferes with the ability of IL-6 alone or in combination with sIL-6R to induce nuclear translocation of GFP-Stat3 in a high-throughput fluorescence microscopy assay we developed to identify small-molecule Stat3 inhibitors (6). The results of this study (**Appendix 2**) indicated that Hextend had no effect on the potency of either IL-6 or sIL-6R.

To explore the hypothesis that shed blood but not Hextend contains a factor(s) such as sIL-6R necessary to realize the beneficial effects of IL-6, in the fourth quarter, we examined the collected shed blood for the presence of sIL-6R as a function of time of accumulation in our standard T/HS protocol. Our results (**Appendix 3**) demonstrated that shred blood accumulated sIL-6R over time to concentrations (100 ng/ml) equivalent to those of recombinant hIL-6 expected within the circulation of rats receiving the 3 ug/kg dose, the dose we initially reported to be of benefit (4).

Studies are underway to provide sIL-6R in addition to IL-6 to T/HS resuscitated with Hextend to determine if sIL-6R is the "missing factor."

Task 1B. _Determine the effect of IL-6 on survival in the rat Lap/HS model._

Subtask IB1. _Randomly assign rats to the Lap/HS/Hex/P or Lap/HS/Hex/IL-6 groups and observe for 72 hr and record mortality. (20 rats; timeframe=2 months)_

While the Novartis experiment was being analyzed by the company, we proceeded with an IL-6 survival study in the Rat Lap/HS model in which the shock load was 180 min at target MAP of 35 mm Hg. Eighteen rats were entered into this protocol and randomized to receive either IL-6 (10 ug/kg in 0.1 ml PBS) or PBS alone as a resuscitation adjuvant. Randomization was performed in such a way that the animal surgeon was blinded to the resuscitation adjuvant each animal received. The PI broke the code on the randomization after 18 rats had been studied.

The results demonstrated that while mortality in the IL-6-treated arm (n=10) was 20%, mortality in the placebo arm was only 25%, lower than the 50% expected.

We have suspended further entry of rats into this protocol until we achieve clarity regarding why Hextend resuscitation blunts the benefit of IL-6.

Task 1C. ***Determine the effect of IL-6 on left ventricular contractile function, apoptosis within the heart, lung, liver, kidney, kidney and leukocytes, injury and inflammation within the lung and liver and mitochondrial dysfunction within liver and hearts.***

To lay the foundation for the mitochondrial portion of these studies, we performed a preliminary study to evaluate the potential acute effect of IL-6 administration on mitochondrial function. Three pairs of rats were administered either 10 ug/kg IL-6 (ip) or vehicle (PBS) and one hour later the animals were sacrificed, organs (heart, kidney, liver and lung) harvested, and mitochondria were isolated. The isolated mitochondria were assayed for respiration by polarography, for respiratory chain activities by spectrophotometry, and for relative mitochondrial reactive oxygen species (ROS) levels by measuring native and total reduced aconitase activities. The results of studies on mitochondria isolated from each of the four tissues of IL-6 treated animals were unchanged compared to control-treated animals.

We have recently gained access to a Seahorse XF24 Analyzer that allows for significantly increased sensitivity for mitochondrial respiration studies. This will allow for the analysis of 20 mitochondrial samples in parallel in a half day, which previously would have taken 3 days. Therefore, mitochondrial analyses of mitochondria from multiple tissues of paired rats subjected to the standard T/HS model with or without IL-6 administration as an adjuvant resuscitative agent will be greatly facilitated. Experiments to optimize the conditions for measuring respiration of mitochondria from rat heart, liver, kidney and lung are currently underway. However, further mitochondrial and other studies listed in this Task await determination of the need for sIL-6R for IL-6 benefit.

Task 1D. ***Determine the effect of Stat3 inhibition with the GQ-ODN T40214 on IL-6-mediated prevention of T-HS-induced mortality, left ventricular contractile dysfunction, organ apoptosis, injury and inflammation and mitochondrial dysfunction.***

This Task awaits completion of Task 1C.

Specific Aim 5. ***To determine if circulating leukocytes can serve as a marker for T-HS-induced apoptosis in T-HS patients (timeframe=16 months).***

Task 5A. ***Amend IRB protocol (timeframe=4 months).***

This Task was accomplished.

Task 5B. ***Isolate peripheral blood leukocytes from T-HS patients upon entry into the standard vs. hypotensive resuscitation protocol study at the time of randomization (time 0) and at 60-minute intervals until the end of surgery then 1 after the end of surgery and 24 hr after randomization (17 patients; timeframe=10 months).***

This Task was accomplished; see below.

Task 5C. ***Perform nucleosome ELISA on leukocyte extracts (timeframe=1 month).***

This Task was accomplished; see below.

Task 5D. _Perform TUNEL of leukocytes (timeframe=1 month)._

Tasks 5B, 5C and 5C were accomplished with the following modifications. Instead of studying only 17 patients, we examined 41 patients. Also, rather than isolating WBC for ELISA and TUNEL at the time of randomization, every hour after randomization, 1 hour after the end of surgery and 24 hours after randomization, we opted to drop the hourly examinations during surgery because of the difficulty in coordinating blood sampling while the patient was undergoing life-saving surgery. Instead, we collected blood for WBC isolation and examinations at the time of randomization, 1 hour after the end of surgery and 24 hour after randomization. Our results demonstrated that there was very little difference in leukocyte apoptosis or any other clinically relevant parameter between these two groups. Consequently, the two group were pooled resulting in the finding [**Appendix 4**; (7)] that the circulating leukocytes of T/HS subjects who survived to hospital discharge without developing any infections had significantly higher nucleosome levels 1-hr post-operative compared to those who did develop an infection (49.8 mU/mg protein versus 19.8 mU/mg protein, p=0.02). This difference persisted when analyzing by specific type of infection. TUNEL staining revealed that 72% of apoptotic cells were PMNs. There were no statistically significant correlations between nucleosome levels and survival. Thus, our results indicated that in patients with hemorrhagic shock, increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection. Previous research has shown that high levels of apoptosis in circulating neutrophils following shock may have a protective effect by preventing neutrophil infiltration and limiting release of harmful oxygen radicals in the tissues. Thus, neutrophil apoptosis may render tissues less susceptible to injury and subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

As noted, our results did not demonstrate an association between leukocyte apoptosis and survival. Consequently, leukocyte apoptosis cannot serve as a robust biomarker for predicting patients who may benefit from IL-6 as a resuscitation adjuvant. Given the results above demonstrating the sensitivity of urinary injury biomarkers in our rat T/HS model, with the agreement of Novartis, we will begin to collect the urine of T/HS patients for measurement of markers of renal injury to establish the best biomarker of renal injury in this patient population to replace leukocyte apoptosis for this purpose. Urine will be collected at the same time points as previously used for blood plus 7 days after randomization.

Thus, all of Aim 5 has been accomplished resulting in a submitted manuscript. We have made good progress in Aim 1. It will be rapidly completed once we established the requirement for sIL-6R to achieve benefit from IL-6 when Hextend is used for resuscitation.

In addition to substantial progress in Aims 1 and 5, we have made additional progress in Aim 4 as outlined below.

Specific Aim 4. To determine the effects of IL-6 on the T-HS-induced apoptosis and inflammation transcriptome within the heart, lung, liver, kidney and leukocytes of rats and swine subjected to T-HS and resuscitation protocols (timeframe=12 months; in Year 2).

Since submission of the proposal in which we presented preliminary data regarding the effect of our standard T/HS protocol on the liver inflammation transcriptome and its beneficial

modulation by IL-6, we have published these findings in *PLoS ONE* [**Appendix 5**; (8)]. In addition, the PI was invited to give a plenary presentation to the 34th Annual Conference on Shock (Norfolk, VA; 06/12/11; "Contributions of Abnormal Proteostasis to Cellular Dysfunction"). This talk and the data presented therein formed the basis for a peer-reviewed manuscript entitled, "Contribution of the Unfolded Protein Response (UPR) to Hepatocyte and Cardiomyocyte Apoptosis and its Prevention in Trauma/Hemorrhagic Shock", submitted to *Nature Cell Death and Disease* on November 29, 2011 [**Appendix 6**; (9)]. This paper reports the first global transcriptome analysis of the UPR in the liver and heart in T/HS; it strongly implicates the non-canonical UPR proteins, heat shock proteins (Hsp) 70 and Hsp40, as modulating hepatocyte apoptosis and mediating protection against apoptosis in response to IL-6. This finding opens new avenues for intervention in the prevention of apoptosis in this setting such as proteostasis modulators. In addition, Stephen Thacker, a talented post-doctoral fellow in the laboratory and first author of the *Nature* submission, has submitted 3 abstracts in the last six months to annual national meetings of the Infectious Diseases Society of America, the Pediatric Academic Society, and the Pediatric Infectious Diseases Society (**Appendix 7**) describing the UPR and it role in alveolar epithelial cell apoptosis in T/HS and its prevention by IL-6.

KEY RESEARCH ACCOMPLISHMENTS:

- We demonstrated that kidney injury accompanies heart, lung and liver injury in rat T/HS.
- Kidney injury was readily detected within the urine 4 hours after the initiating of resuscitation using 7 of 11 analytes tested by Novartis; 4 of these 7 analytes are available for purchase and use in a Luminex bead-based assay system in the Tweardy lab.
- Results demonstrated that heparinized shed blood accumulated sIL-6R over time to concentrations equivalent to those of recombinant hIL-6 expected within the circulation of rats receiving the 3 ug/kg dose, the dose we initially reported to be of benefit (4). Thus, our experiments may have uncovered an essential factor, in addition to IL-6, required for prevention of apoptosis when IL-6 is used as a resuscitation adjuvant.
- While leukocyte apoptosis appears not to be sufficiently robust enough biomarker for predicting mortality in T/HS patients, our results examining this biomarker indicated that in patients with hemorrhagic shock, increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection.
- In the first global analysis of the UPR transcriptome ever performed, we identified two non-canonical UPR modulators, Hsp70 and Hsp40, as potential key modulators of liver and lung apoptosis in T/HS that mediate the beneficial effects of IL-6.

REPORTABLE OUTCOMES:

Manuscripts:

1. Moran, A., Thacker, S.A., Arikan, A.A., Mastrangelo, M.A., Wu, Y., Yu, B., and Tweardy, D.J. 2011. IL-6-mediated activation of Stat3alpha prevents trauma/hemorrhagic shock-induced liver inflammation. *PLOS One* 6:e21449.
2. Morrison, C.A., Moran, A., Huby M.P., Tweardy, D.J., and Carrick, M.M. 2011. Increased Apoptosis of Peripheral Blood Neutrophils is Associated with Reduced Risk of Infection in Trauma Patients with Hemorrhagic Shock. *Journal of Trauma* Submitted.

3. Thacker, S.A., and Tweardy, D.J. 2011. Contribution of the Unfolded Protein Response to Hepatocyte and Cardiomyocyte Apoptosis and its Prevention in Trauma/Hemorrhagic Shock. *Nature Cell Death and Disease* Submitted.

Abstracts:
1. Thacker S, Moran A, Lionakis M, Mastrangelo MA, Halder T, Huby MP, Wu Y, and Tweardy DJ. Protect the cell to protect the host, linking the unfolded protein response to alveolar epithelial cell apoptosis and susceptibility to Pseudomonas aeruginosa pneumonia in the injured host. Submitted to the Annual Meeting of the Infectious Diseases Society of America 2011.
2. Thacker SA, Moran A, Huby M and Tweardy DJ. Contribution of Heat Shock Proteins 70 and 40 to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. Submitted to the Annual Meeting of the Pediatric Academic Society 2012.
3. Thacker SA, Moran A, Huby M and Tweardy DJ. Contribution of Heat Shock Proteins 70 and 40 to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock. Submitted to the Annual Meeting of the Pediatric Infectious Diseases Society 2012.

National Presentations:

Plenary Presentation to the 34th Annual Conference on Shock in Norfolk, VA on 06/12/11 entitled, "Contributions of Abnormal Proteostasis to Cellular Dysfunction".

CONCLUSIONS:

We have performed a global transcriptome analysis of the UPR in the liver and heart in T/HS that strongly implicates Hsp70 and Hsp40 as modulating hepatocyte apoptosis and mediating protection against apoptosis in response to IL-6. This finding opens new avenues for intervention in the prevention of apoptosis in this setting such as proteostasis modulators.

We demonstrated in patients with hemorrhagic shock that increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection. Previous research has shown that high levels of apoptosis in circulating neutrophils following shock may have a protective effect by preventing neutrophil infiltration and limiting release of harmful oxygen radicals in the tissues. Thus, neutrophil apoptosis may render tissues less susceptible to injury and subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

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3. Moran, A., Akcan Arikan, A., Mastrangelo, M.A., Wu, Y., Yu, B., Poli, V., and Tweardy, D.J. 2008. Prevention of trauma and hemorrhagic shock-mediated liver apoptosis by activation of stat3alpha. *Int J Clin Exp Med* 1:213-247.
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7. Morrison, C.A., Moran, A., Huby, M.P., Tweardy, D.J., and Carrick, M.M. 2011. Increased Apoptosis of Peripheral Blood Neutrophils is Associated with Reduced Risk of Infection in Trauma Patients with Hemorrhagic Shock. *Journal of Trauma* Submitted.
8. Moran, A., Thacker, S.A., Arikan, A.A., Mastrangelo, M.A., Wu, Y., Yu, B., and Tweardy, D.J. 2011. IL-6-mediated activation of Stat3alpha prevents trauma/hemorrhagic shock-induced liver inflammation. *PLOS One* 6:e21449.
9. Thacker, S.A., and Tweardy, D.J. 2011. Contribution of the Unfolded Protein Response to Hepatocyte and Cardiomyocyte Apoptosis and its Prevention in Trauma/Hemorrhagic Shock. *Nature Cell Death and Disease* Submitted.

APPENDICES:

Appendix 1: Novartis report on renal injury urine biomarkers in rat T/HS model *(pgs. 12 - 32)*

Appendix 2: Tweardy lab experiment demonstrating no inhibitory effect of Hextend on IL-6 or sIL-6 activity *(pg. 33)*

Appendix 3: Tweardy lab experiment demonstrating accumulation of sIL-6R in heparinized shed blood in rat T/HS model *(pgs. 34 - 37)*

Appendix 4: Morrison et al manuscript submitted *Journal of Trauma (pgs. 38 - 55)*

Appendix 5: Moran et al manuscript published in *PLoS One (pgs. 56 - 64)*

Appendix 6: Thacker and Tweardy manuscript submitted to *Nature Cell Death and Disease (pgs. 65 - 96)*

Appendix 7: Thacker et al abstracts to annual meetings of IDSA, PAS, and PIDS *(pgs. 97, 98, 99)*

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ILS969: Biomarker Analysis

Eric Johnson, David Kagan, Neeta Shenoy, and Aaron Nelson (BTT/iTox)

June 2011

NOVARTIS

Study Design:

SHAM **6 rats**	4 12h 18h 24h 36h 42h 48h
HS **6 rats**	4 12h 18h 24h 36h 42h 48h
HS-R **Low** **6 rats**	4 12h 18h 24h 36h 42h 48h
HS-R **Med** **6 rats**	4 12h 18h 24h 36h 42h 48h
HS-R Hi **6 rats**	4 12h 18h 24h 36h 42h 48h

30 rats = 275 samples
urine & serum

HS: Hemorrhagic Shock

2405 data points

MSD

kidney

Clusterin

Cystatin C

β2M

αGST
GSTYb1
RPA1

Albumin
NGAL
Kim1
OPN

SGX
cardio

cTnI

Clin
Chem
Anal

AST
ALP
ASP
Bilirubin
Creatinine

NOVARTIS

Heat Map (Not normalized to creatinine)

Shock

IL6 low

IL6 med

IL6 hi

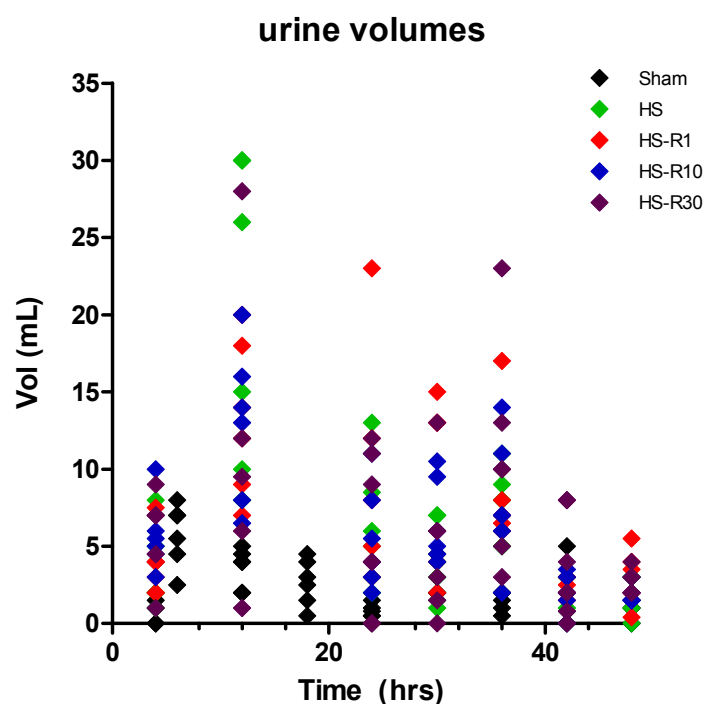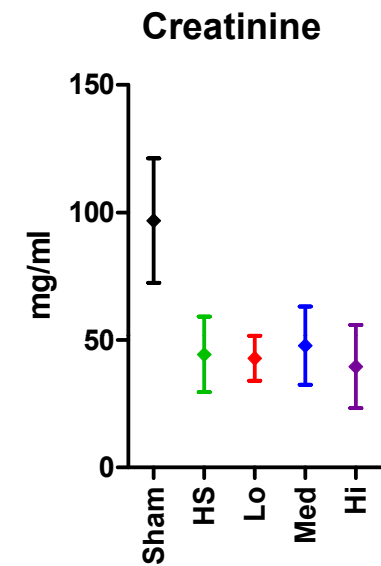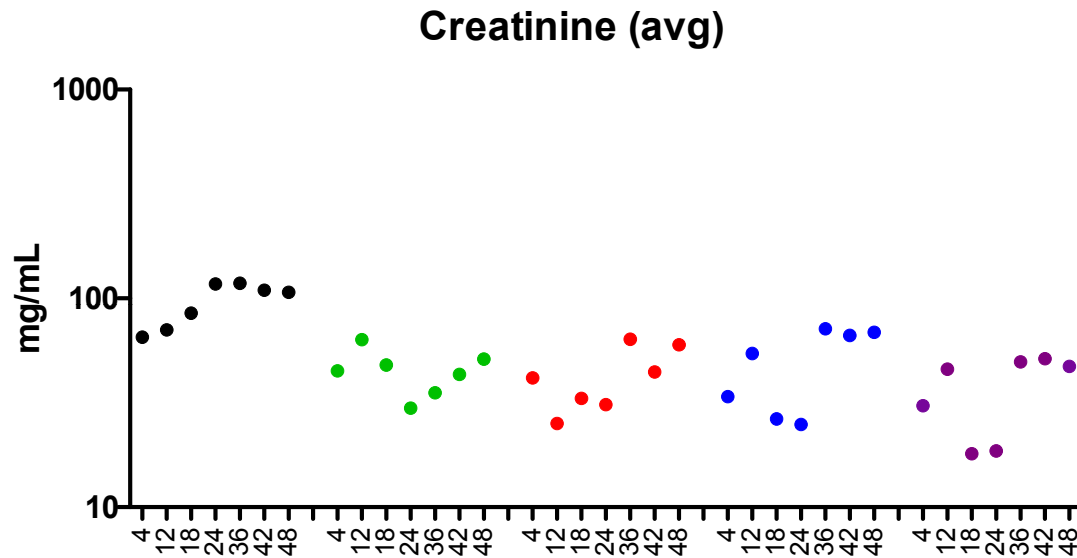sham

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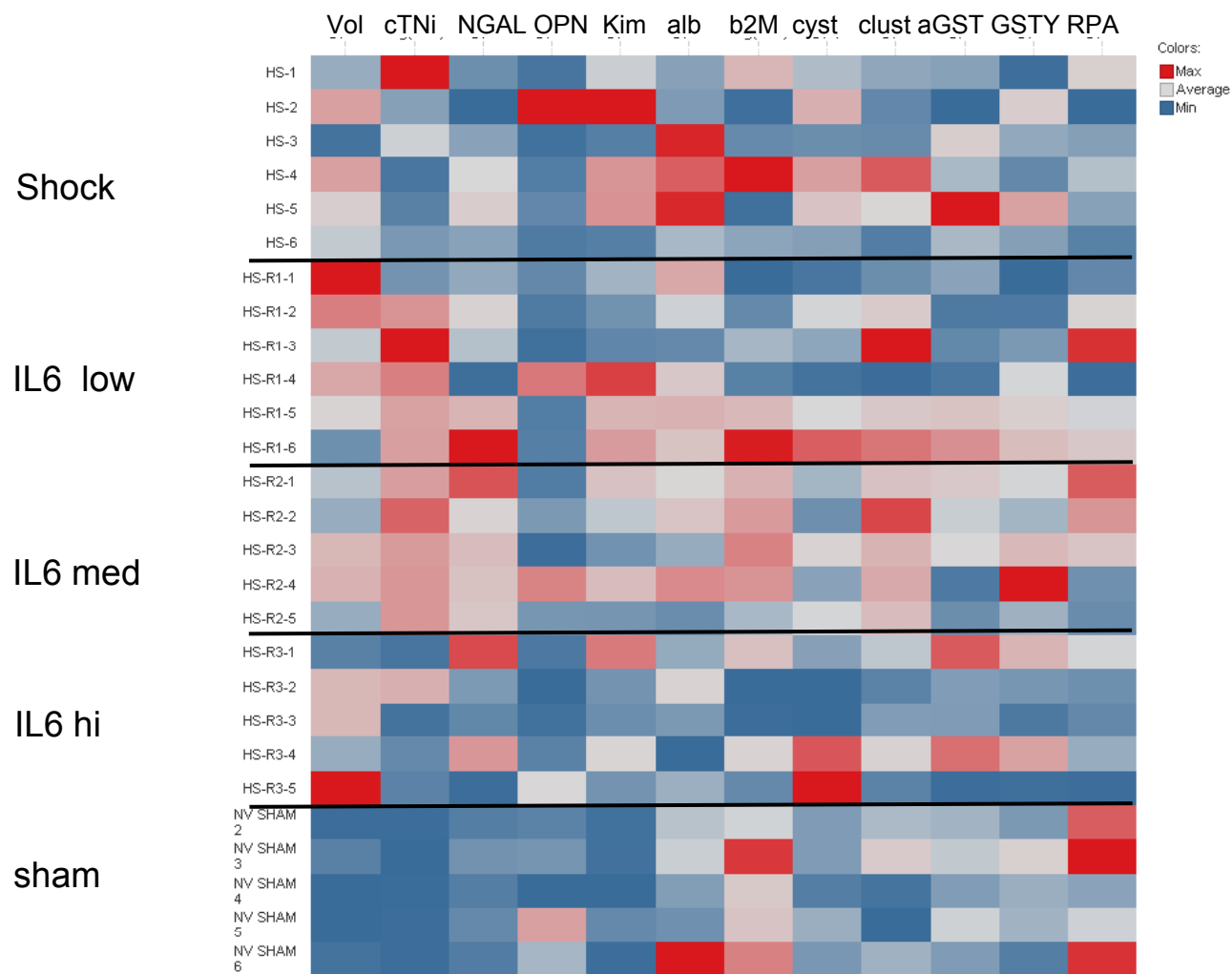
Volumes of Urine Samples Collected

Due to large variations in the sample volumes, data is also represented as a ratio with urinary creatinine

Urinary creatinine was used since there were no matching serum samples

urine volumes

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Urinary Creatinine

Creatinine (avg)

Creatinine

Data indicate average of all animals at all time points +/- SD

Unpaired t test				
Column A	**Sham**	**HS**	**HS**	**HS**
Column B	**HS**	**Lo**	**Med**	**Hi**
Table Analyzed	**Creatinine**			
Are means signif.	Yes	No	No	No
Are variances	No	No	No	No

Data provided by Gordon Turner and Jeff Brown (CVM)

low creatinine levels can result from over-hydration or diluted urine samples

In this analysis, since all urine samples were diluted to the same extent, the low creatinine levels in the HS group as compared to the sham group is likely due to over-hydration

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Kidney Biomarkers Elevated in HS Group

Biomarker	Damage	Description
Clusterin	Tubular *and Glomerular*	Conserved protein induced during tissue injury or remodeling
KIM-1	Tubular (proximal)	Type 1 transmembrane glycoprotein found on CD4+ T cells and renal proximal tubule epithelial cells. High levels found due to ischemic damage
Cystatin C	Glomerular	Extracellular inhibitor of Cys proteases normally expressed in vascular wall smooth muscle cells
β2M	Tubular (Proximal) and Glomerulus	Small cell surface protein shed into blood and normally reabsorbed by proxima tubule. High levels result from lack of reabsorption due to renal failu
Albumin	Glomerular, tubular	Albumin is the most abundant serum protein that acts as a transport protein for hemin and fatty acids. It is produced in the liver and secreted in the bloodstream. Damage to the kidney can lead to albuminuria, secretion of albumin into the urine.
GSTYb1	Tubular (distal)	GSTYb1 has been shown to leak from distal tubule cells into the urine in response to injury.
NGAL	Tubular (proximal)	Expressed in kidney cells as protective mechanism during the inflammarory response
OPN	Tubular injury and Loop of Henle	Osteopontin (OPN) is a macrophage chemotactic and adhesion molecule and has been shown to play a role in glomerular and tubulointerstitial injury in several kidney disease models.
RPA1	collecting duct	Renal Papillary Antigen 1 (RPA-1) is the first urinary biomarker for injury to the luminal epithelial cell of the collecting duct
GSTα	Tubular (proximal)	Contributes to detoxification of wide range of cmpds including carcinogens therapeutic drugs, and productd of oxidative stress

Of the 10 kidney biomarkers measured, the levels of 8 analytes were significantly different in the HS group as compared to the sham as analyzed by Student t test

HS group sustained injury to glomerulus and proximal tubule based on the biomarker data
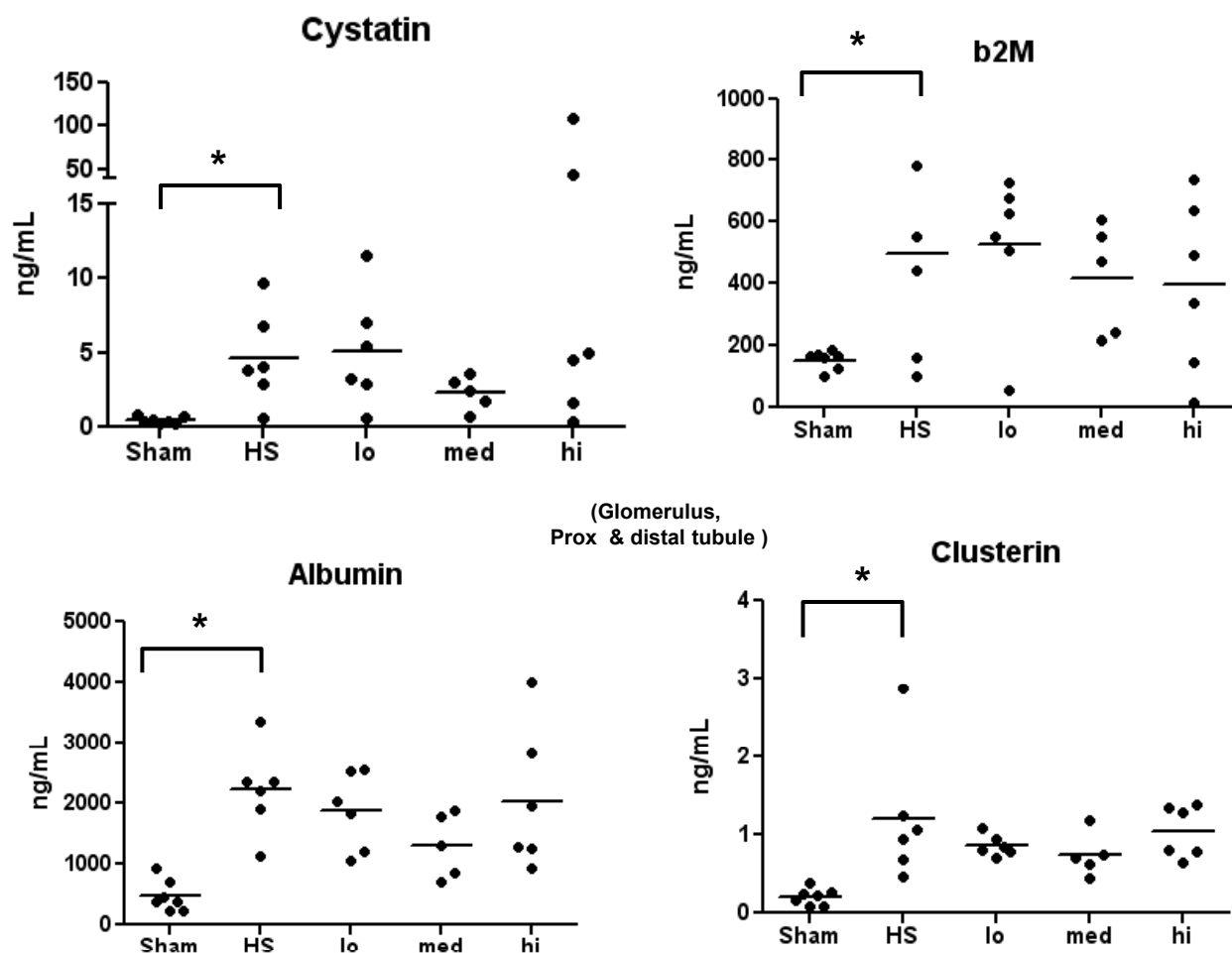
NOVARTIS

Kidney Biomarkers with Differences in Levels between Sham and HS Groups (Normalized to Urinary Creatinine)
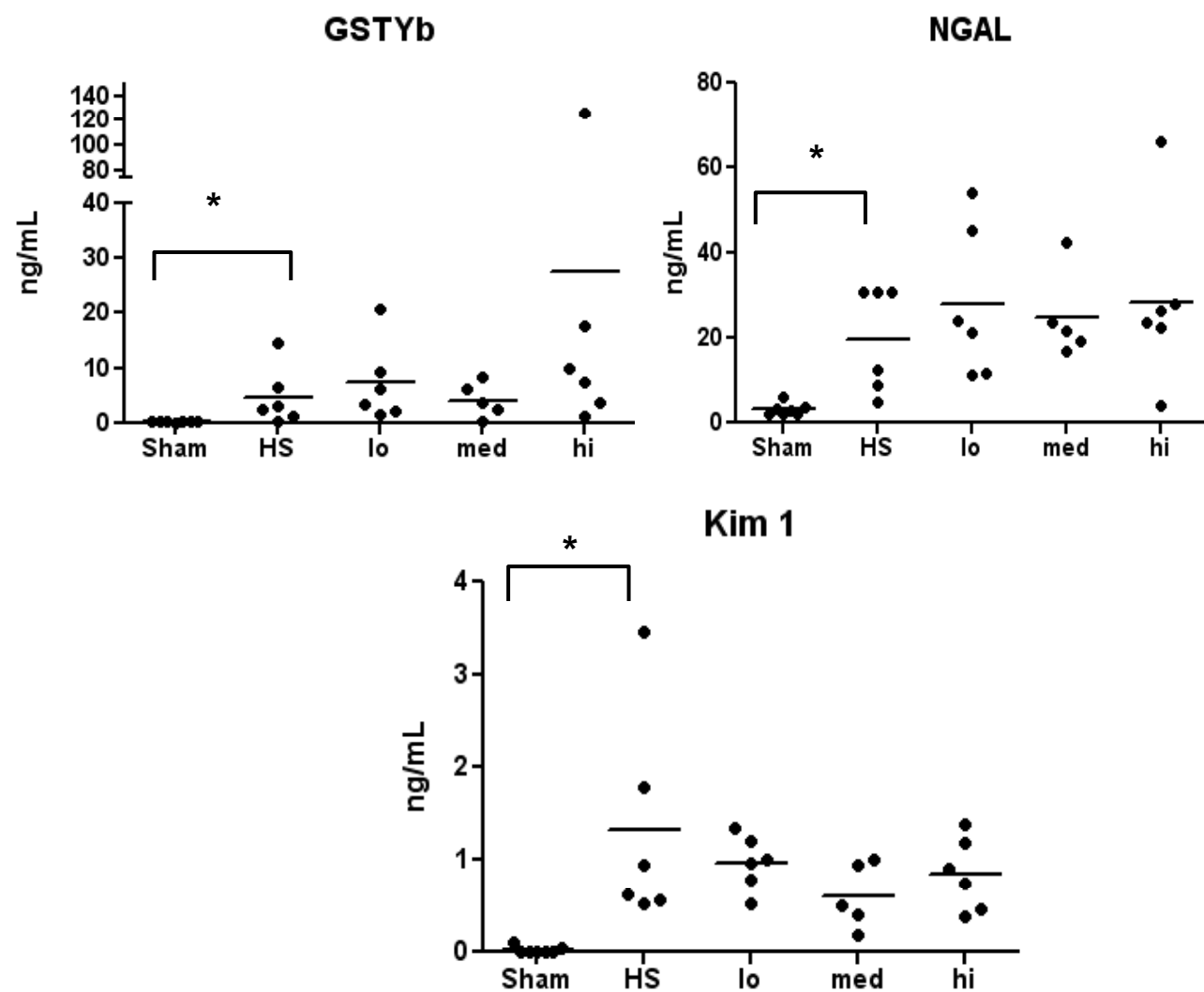
HS groups shows significant elevation in urinary kidney biomarkers that are indicative of damage to the **proximal tubule** as compared to the sham group

ILS969 treatment does not alter the levels of these biomarkers in urine

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Kidney Biomarkers with Differences in Levels between Sham and HS Groups (Normalized to Urinary Creatinine)

Cystatin

b2M

(Glomerulus, Prox & distal tubule)

Albumin

Clusterin

HS groups shows significant elevation in urinary kidney biomarkers that are indicative of damage to the **tubule and glomerulus** as compared to the sham group

ILS969 treatment does not alter the levels of these biomarkers in urine
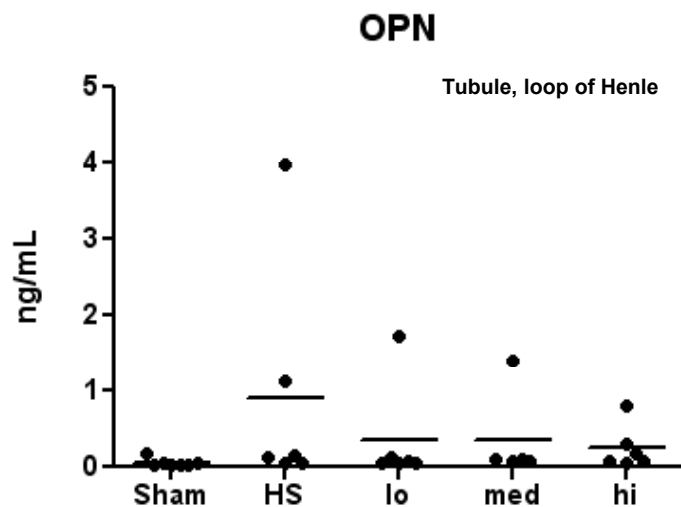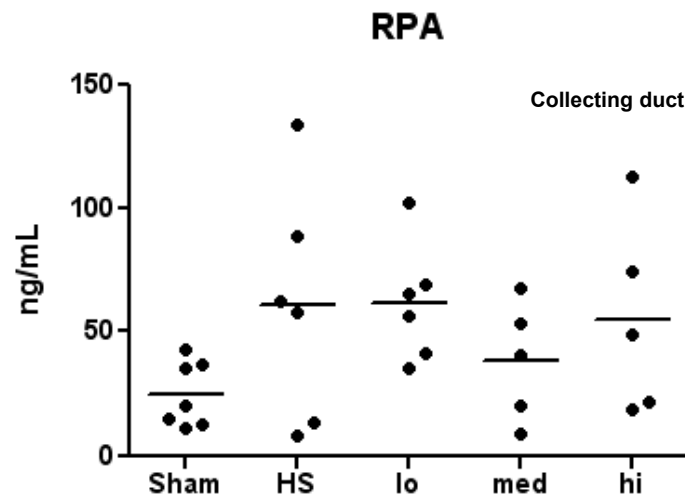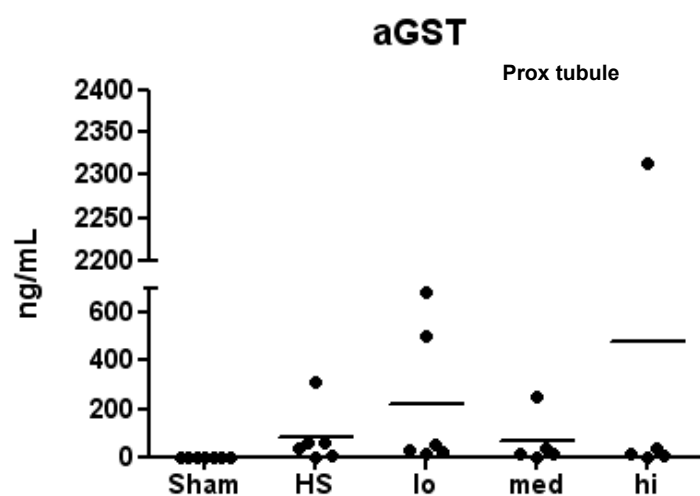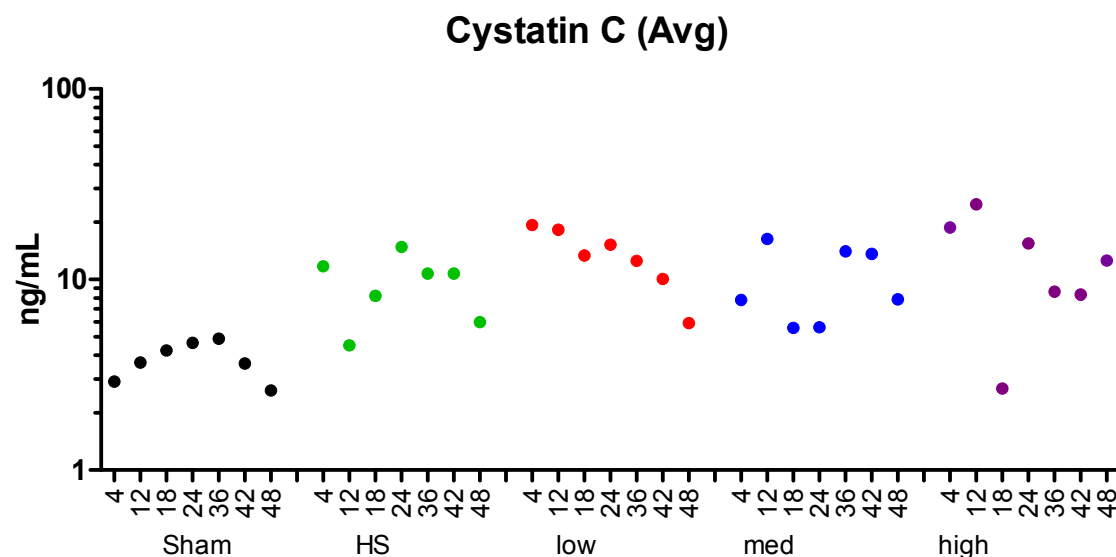
NOVARTIS

Kidney Biomarkers with No Differences in Levels between Sham and HS Groups (Normalized to Urinary Creatinine)

HS groups do not show significant elevation in these urinary kidney biomarkers as compared to the sham group

NOVARTIS

Cystatin C (glomerulus/proximal tubule)

Cystatin C (Avg)

Normalized to Urinary Creatinine
CystatinC
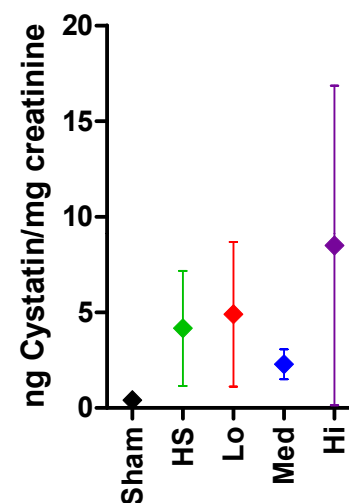
*Data indicate average of all animals at
all time points +/- SD*

Unpaired t test		Sham	HS	HS	HS
		HS	Lo	Med	Hi
Table Analyzed		Cystatin C			
Are means signif. different?		Yes	No	No	No
Are variances signif.different?		Yes	No	No	No

**ILS969 treatment did not alter
the levels of Cystatin C as
compared to the HS group**

NOVARTIS

Kim1 (proximal tubule)
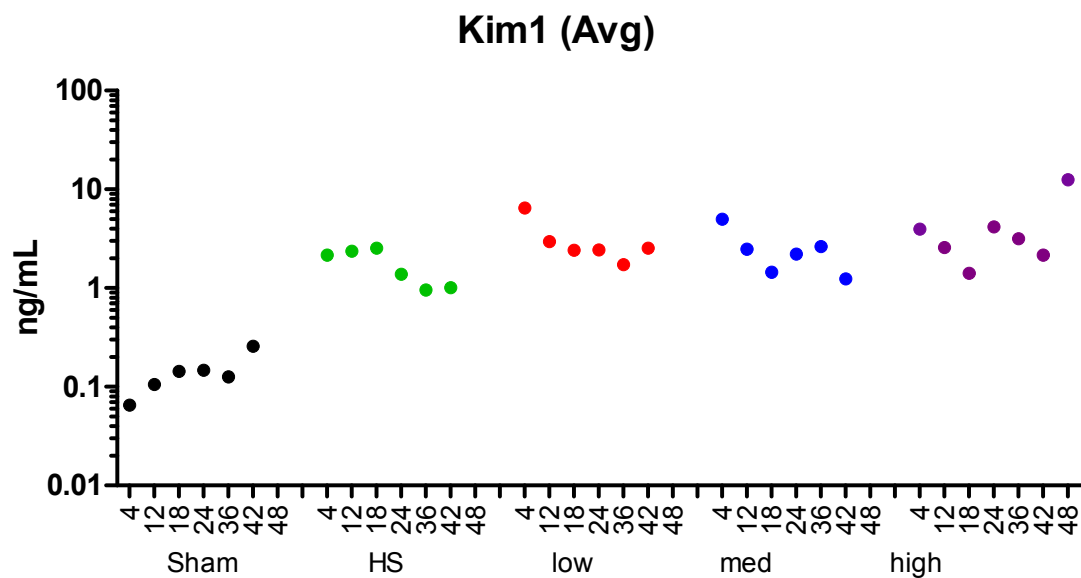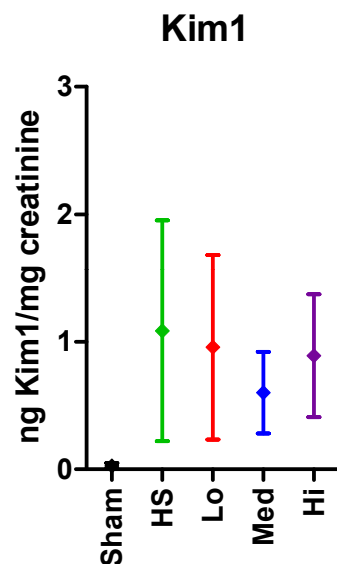
Kim1 (Avg)

Normalized to Urinary Creatinine

Kim1

Data indicate average of all animals at all time points +/- SD

ILS969 treatment did not alter the levels of KIM1 as compared to the HS group

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Table Analyzed	Kim1			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	No

Albumin (glomerulus)

Albumin (Avg)

ng/ml

100000 — 10000 — 1000

Sham | HS | low | med | high

(x-axis time points: 4, 12, 18, 24, 36, 42, 48)

Normalized to Urinary Creatinine

Albumin

ng Albumin/mg creatinine

4000 — 3000 — 2000 — 1000 — 0

Sham | HS | Lo | Med | Hi

Data indicate average of all animals at all time points +/- SD

ILS969 treatment did not alter the levels of albumin as compared to the HS group
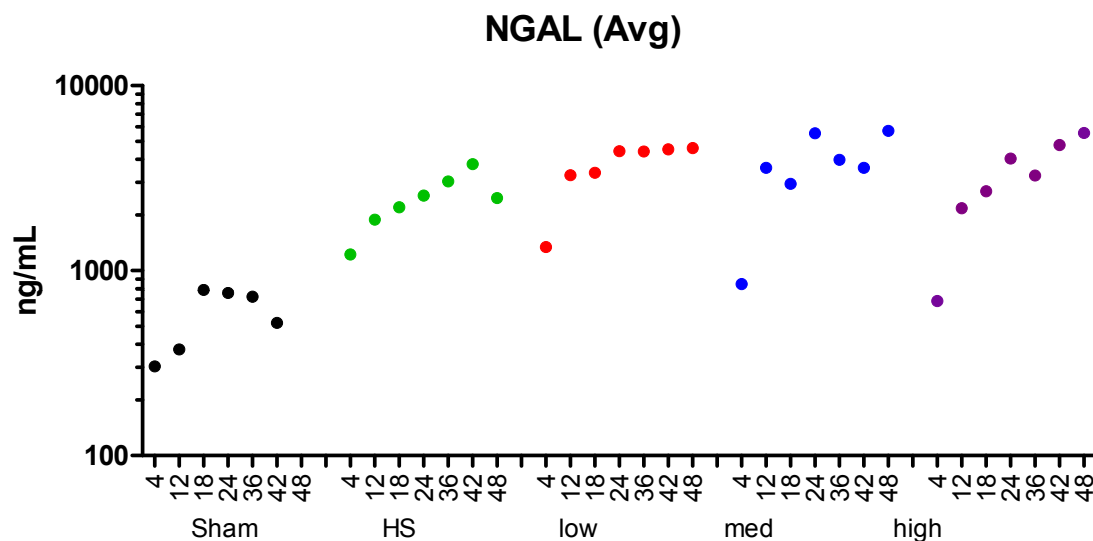
Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Analyte	Albumin			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	Yes	Yes	No

NOVARTIS

NGAL (proximal tubule)

NGAL (Avg)

Normalized to Urinary Creatinine

NGAL

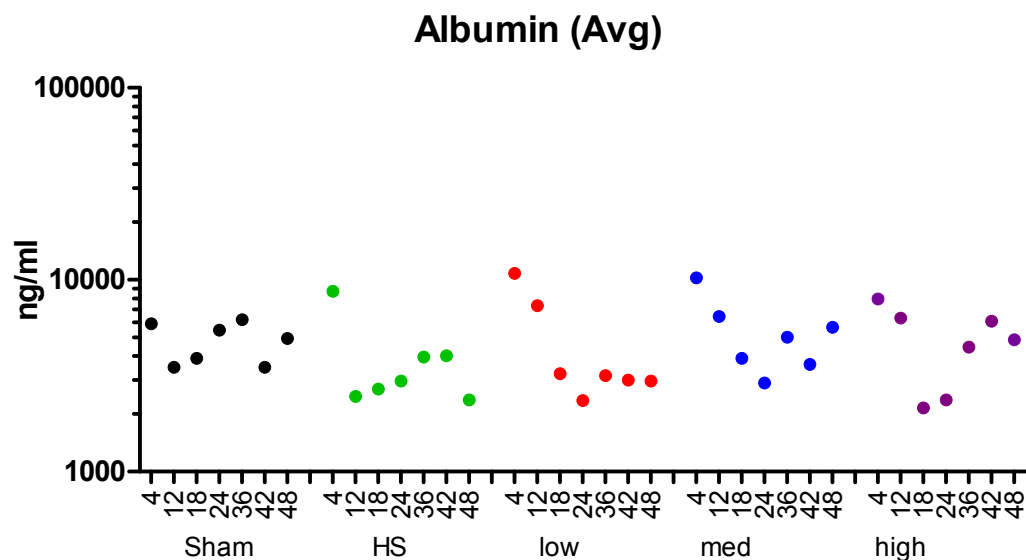*Data indicate average of all animals at all time points +/- SD*

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Table Analyzed	NGAL			
Are means signif. different?	Yes	No	No	No
Are variances signif. different?	Yes	Yes	Yes	No
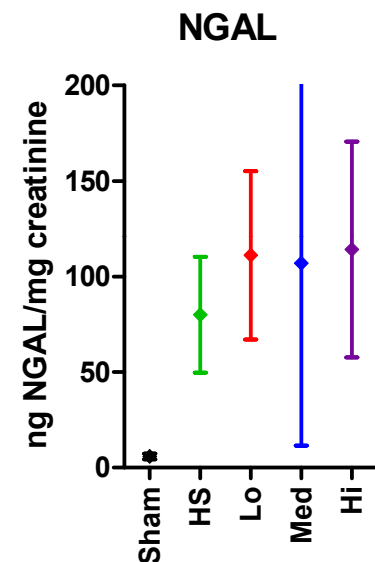
ILS969 treatment did not alter the levels of NGAL as compared to the HS group

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Clusterin (proximal tubule)
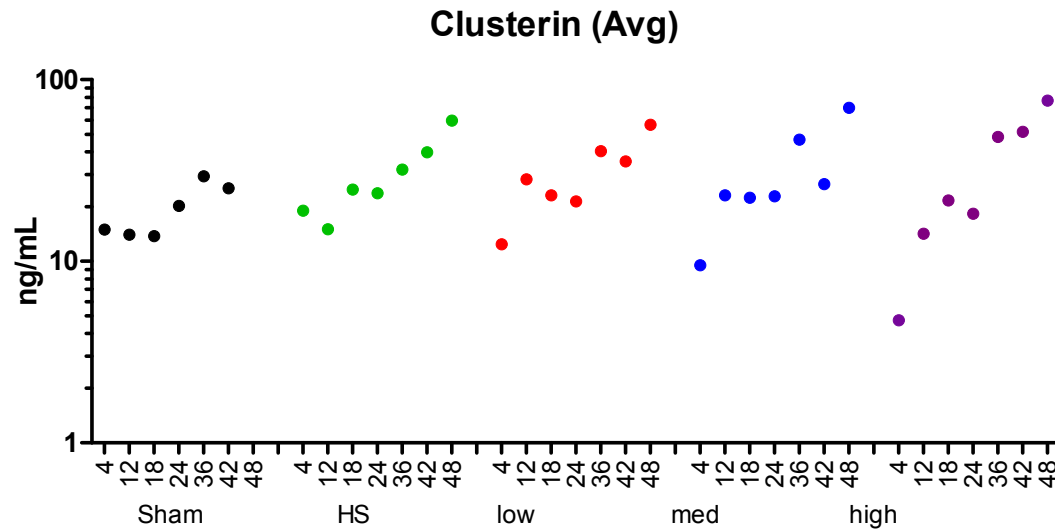
Clusterin (Avg)

Normalized to Urinary Creatinine

Clusterin

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Table Analyzed	Clusterin			
Are means signif. different?	Yes	No	No	No
Are variances signif. different?	Yes	No	No	No
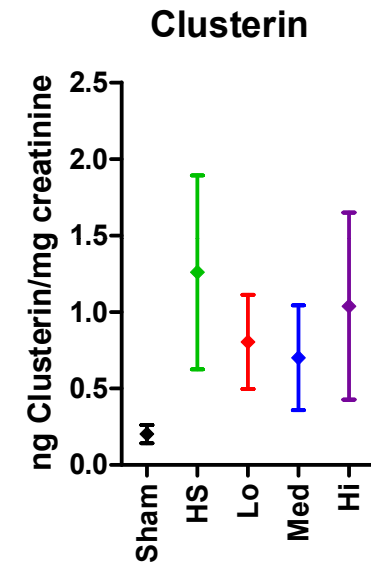
Data indicate average of all animals at all time points +/- SD

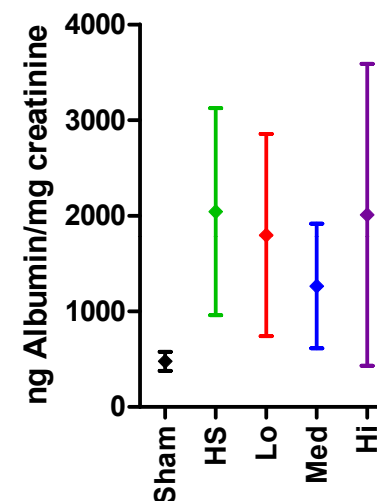**ILS969 treatment did not alter the levels of clusterin as compared to the HS group**

NOVARTIS

GSTYb1 (distal tubule)
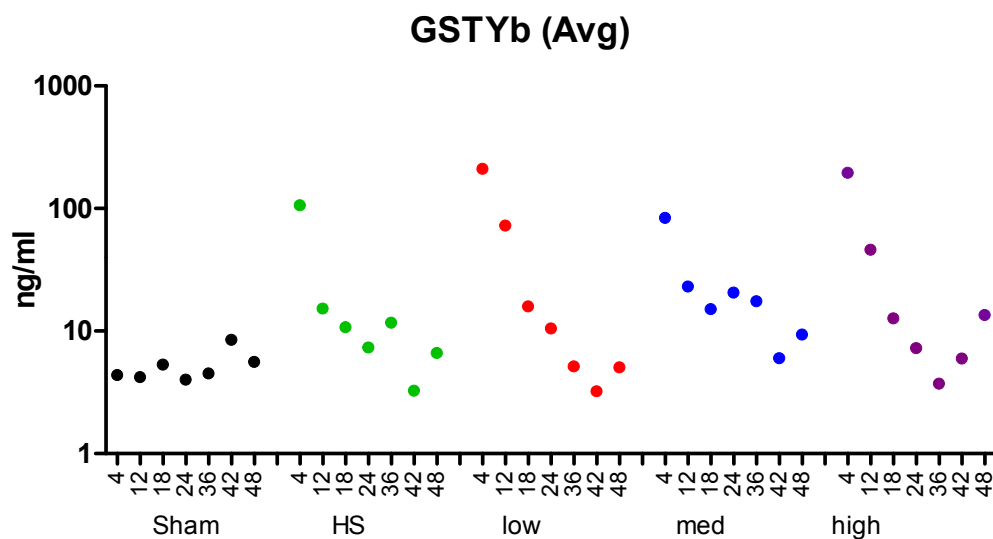
GSTYb (Avg)

Normalized to Urinary Creatinine

GSTYb

Data indicate average of all animals at all time points +/- SD

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Table Analyzed	GSTYb			
Are means signif. different?	Yes	No	No	No
Are variances signif. different?	Yes	No	No	Yes
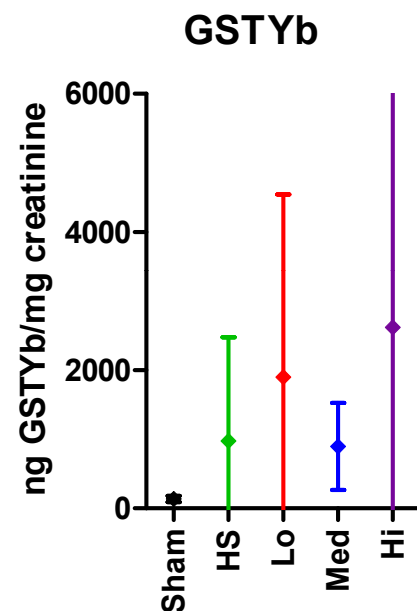
ILS969 treatment did not alter the levels of GSTYb as compared to the HS group

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β2Microglobulin (glomerulus)
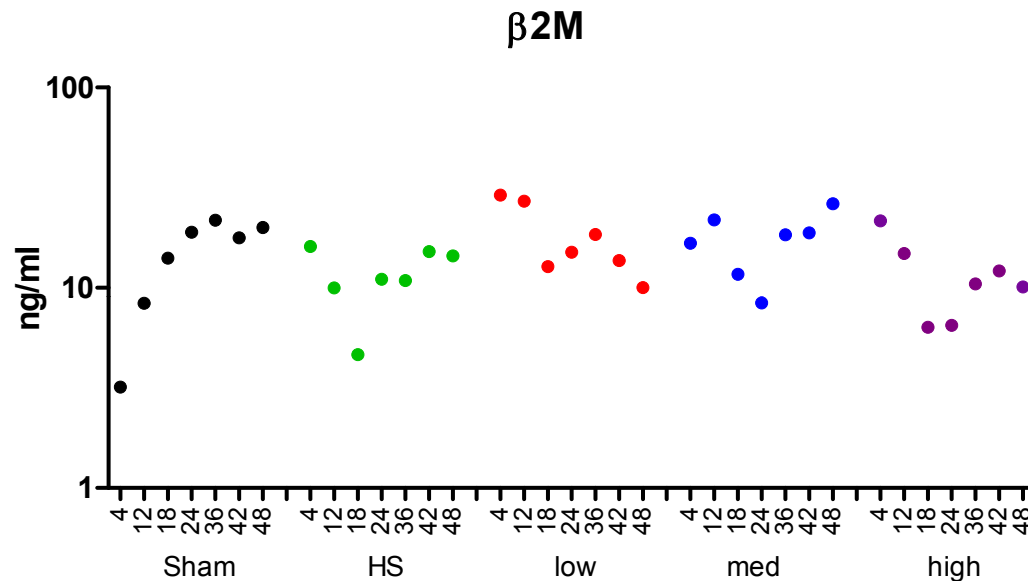
β2M

Normalized to Urinary Creatinine

b2M

Data indicate average of all animals at all time points +/- SD

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Table Analyzed	β2M			
Are means signif. different?	Yes	No	No	No
Are variances signif. different?	Yes	No	No	No
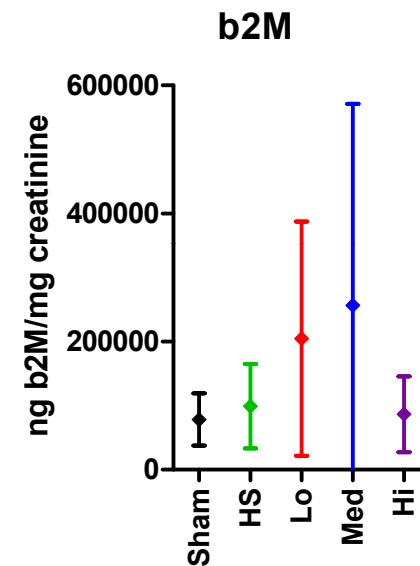
ILS969 treatment did not alter the levels of β2M as compared to the HS group

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Summary: Biomarkers of Kidney Injury in HS

Unpaired t test	Sham	HS	HS	HS
	HS	Lo	Med	Hi
Analyte	Albumin			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	Yes	Yes	No
Table Analyzed	Clusterin			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	No
Table Analyzed	Cystatin C			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	No
Table Analyzed	Kim1			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	No
Table Analyzed	NGAL			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	Yes	Yes	No
Table Analyzed	GSTYb			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	Yes
Table Analyzed	β2M			
Are means signif. different?	Yes	No	No	No
Are variances signif.different?	Yes	No	No	No

With the exception of GSTα, RPA, and osteopontin, all kidney biomarkers were significantly increased in HS group as compared to the sham

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cTnI (Cardiac)

cTnI levels were significantly increased in serum samples from HS group as compared to the sham treated animals

ILS969 treatment did not affect the levels of cTnI as compared to the HS group.

Trends in slight increases (not significant) in cTnI levels at the highest dose of ILS969
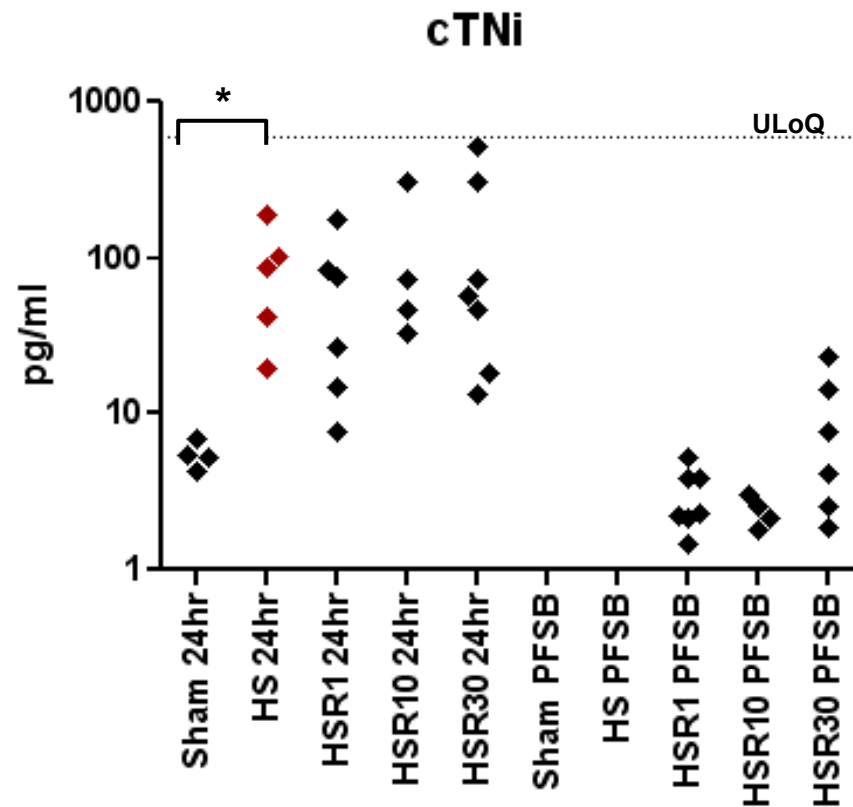
PFSB: Plasma From Spent Blood

Alanine aminotransferase, Aspartate aminotransferase, and Alkaline phosphatase (Liver Injury)

Levels of liver enzymes were significantly increased in serum samples from HS group as compared to the sham treated animals

ILS969 treatment did not affect the levels of these proteins as compared to the HS group

Data provided by Gordon Turner and Jeff Brown (CVM)

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Biomarkers for Liver Injury: Statistics

	HS 24hr HSR1 24hr	HS 24hr HSR10 24hr	HS 24hr HSR30 24hr
Table Analyzed	**cTNi**	**cTNi**	**cTNi**
Are means signif. different? (P < 0.05)	No	No	No
Are variances significantly different?	No	No	No
Table Analyzed	**AST**	**AST**	**AST**
Are means signif. different? (P < 0.05)	No	No	No
Are variances significantly different?	No	No	Yes
Table Analyzed	**ALP**	**ALP**	**ALP**
Are means signif. different? (P < 0.05)	**Yes**	No	No
Are variances significantly different?	**Yes**	No	No
Table Analyzed	**ALT**	**ALT**	**ALT**
Are means signif. different? (P < 0.05)	No	No	No
Are variances significantly different?	**Yes**	No	Yes

U NOVARTIS

Conclusions:

Of the 10 kidney biomarkers measured, the levels of 8 analytes (KIM1, NGAL, GSTYb, β2M, albumin, cystatin C, and clusterin) were significantly different in the HS group as compared to the sham as analyzed by Student t test . There was no significant changes in the levels of the kidney biomarkers upon treatment with ILS96 (compared to the HS group)
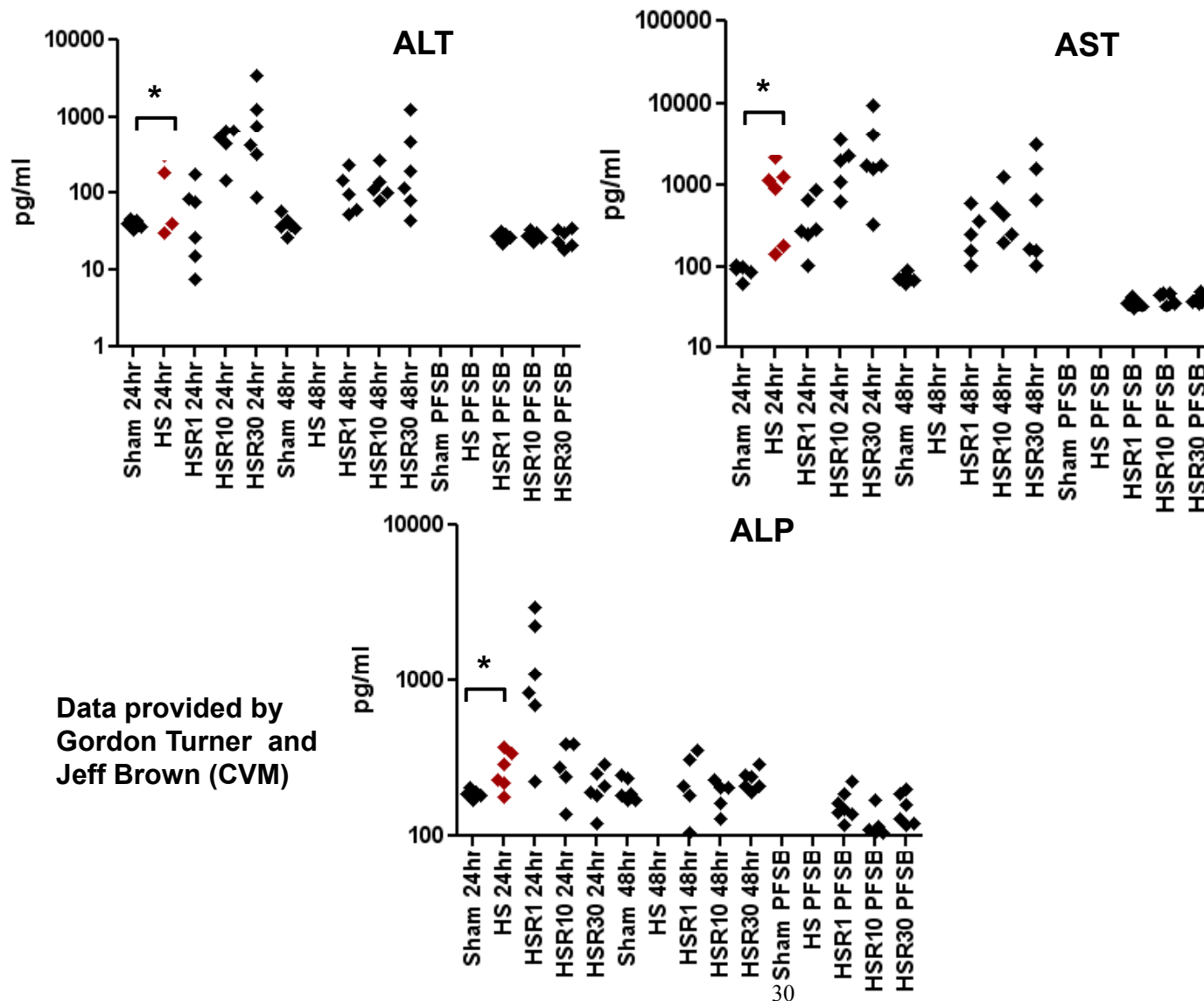
cTnI levels were significantly increased in serum samples from HS group as compared to the sham treated animals. ILS969 treatment did not affect the levels of cTnI as compared to the HS group

Levels of ALT, AST, and AST were significantly different in the HS group as compared to the sham group
There was no significant changes in the levels of the liver biomarkers upon treatment with ILS96 (compared to the HS group). However, there were trends in slight increases (not significant) in ALT and AST levels at the highest dose of ILS969

These data suggest that ILS969 had little or no efficacy as a therapeutic in this study of hemorrhagic shock

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% Hextend ▼	50 ng/ml	100 ng/ml	150 ng/ml	◄IL6/IL6SR
Unstimulated	8.15	3.67	4.50	
0.1	100.00	100.00	100.00	
0.3	103.42	101.99	106.21	
1	107.79	100.38	102.79	
3	107.14	93.01	101.70	
10	102.88	93.90	94.59	
30	87.20	111.53	94.94	

Effect of Increasing Hextend on STAT3 Alpha Nuclear Translocation
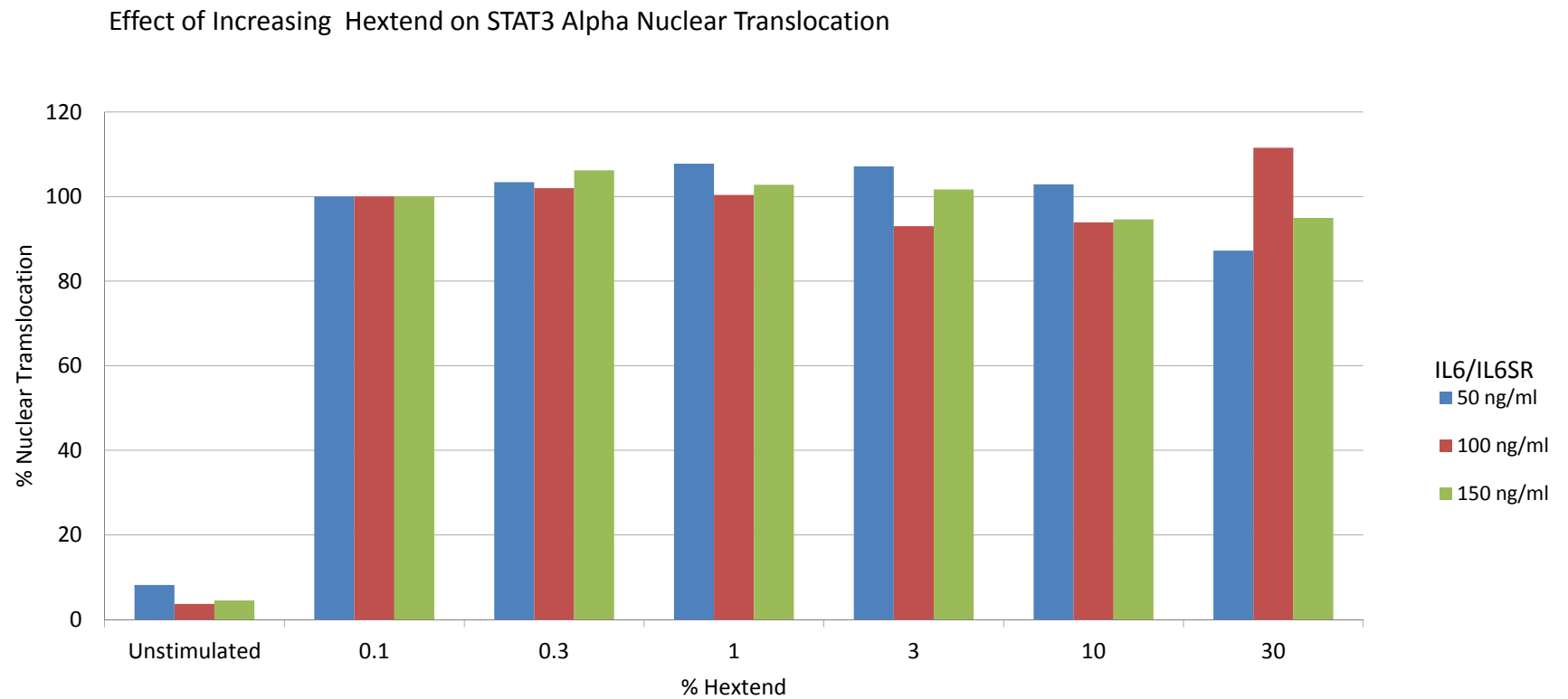
Soluble IL-6 ELISA
Purpose and Sample Procurement

- **Purpose:**
  - To determine if soluble IL-6 receptors are forming in the shed blood

- **Sample Procurement:**
  - Aliquots of pooled heparinized shed blood were collected from 3 rats on 3 different days every 45 minutes
    - 0, 45, 90, 135, and 180 minutes
  - The aliquots were centrifuged at 1000 g for 20 minutes
  - The supernatant was collected and frozen @ -20° C

Soluble IL-6 ELISA Procedure

- Kit was removed from the refrigerator and allowed to warm to room temp for 30 minutes
- The standard curve was prepared
  - 50, 25, 12.5, 6.3, 3.1, and NSB
- The samples were diluted
  - 120 µL diluent + 30 µL sample
- Standard and samples were plated in duplicate and incubated @ 37° C for 30 minutes
- Plate was washed 3X with 1X wash buffer
- Incubate 50 µL/well HRP conjugated antibody @ 37° C for 30 minutes
- Plate was washed 3X with 1X wash buffer
- Incubate 50 µL/well of each chromogen A and B @ 37° C for 15 minutes
- Add 50 µL/well of stop solution and read @ 450 nm

Soluble IL-6 Std Curve
(BG Subtracted)

Soluble IL-6 Receptor Standard
(BG Sub)

$y = 0.012x - 0.027$
$R^2 = 0.9986$

Corrected OD @ 450 nm — Concentration (ng/mL)
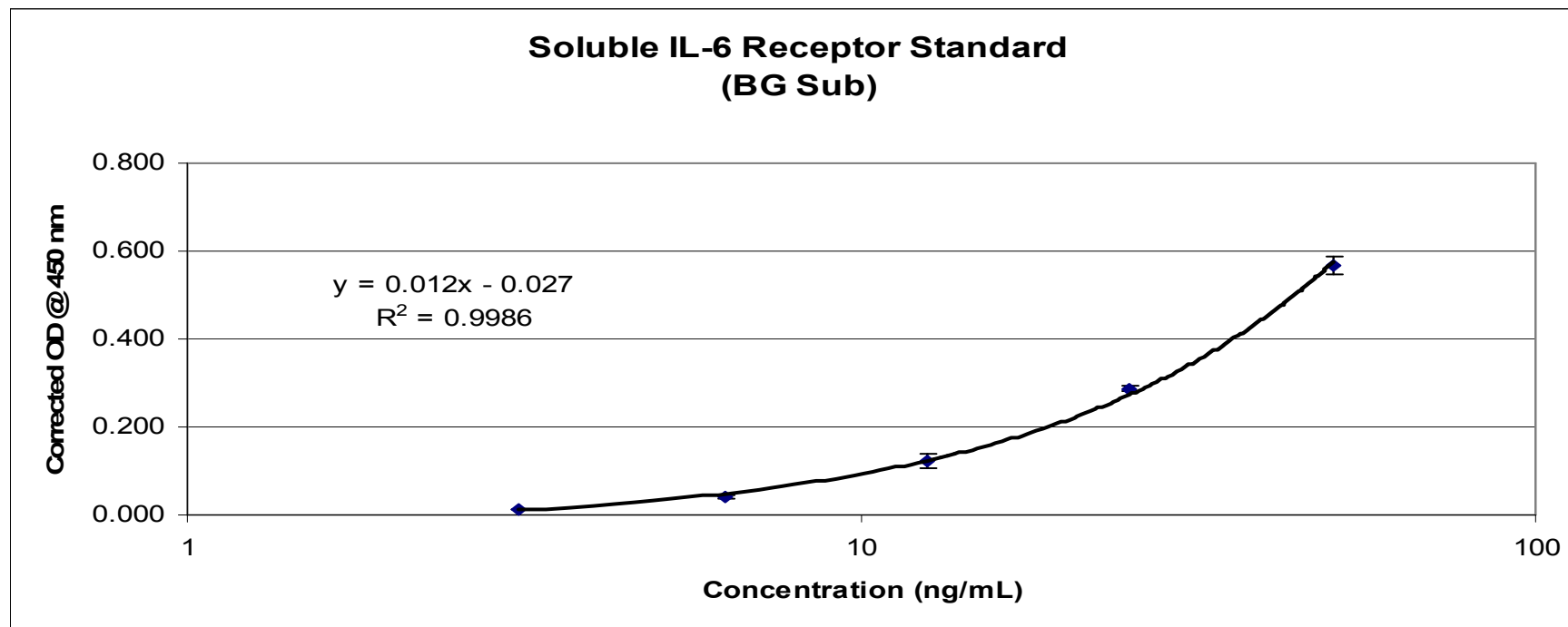
Rat Soluble IL-6 Std Curve (ng/mL)					
	3.1	**6.3**	**12.5**	**25**	**50**
Bg Sub Mean	0.011	0.041	0.123	0.288	0.568
Stdev	0.001	0.006	0.016	0.006	0.019
%CV	0.2	1.5	3.4	0.9	2.1
T-Test, BG	0.5868	0.1991	0.0255	0.0221	0.0012

Concentration Soluble IL-6 Receptor in All 3 Rats

Concentration of Soluble IL-6 Receptor in All 3 Rats
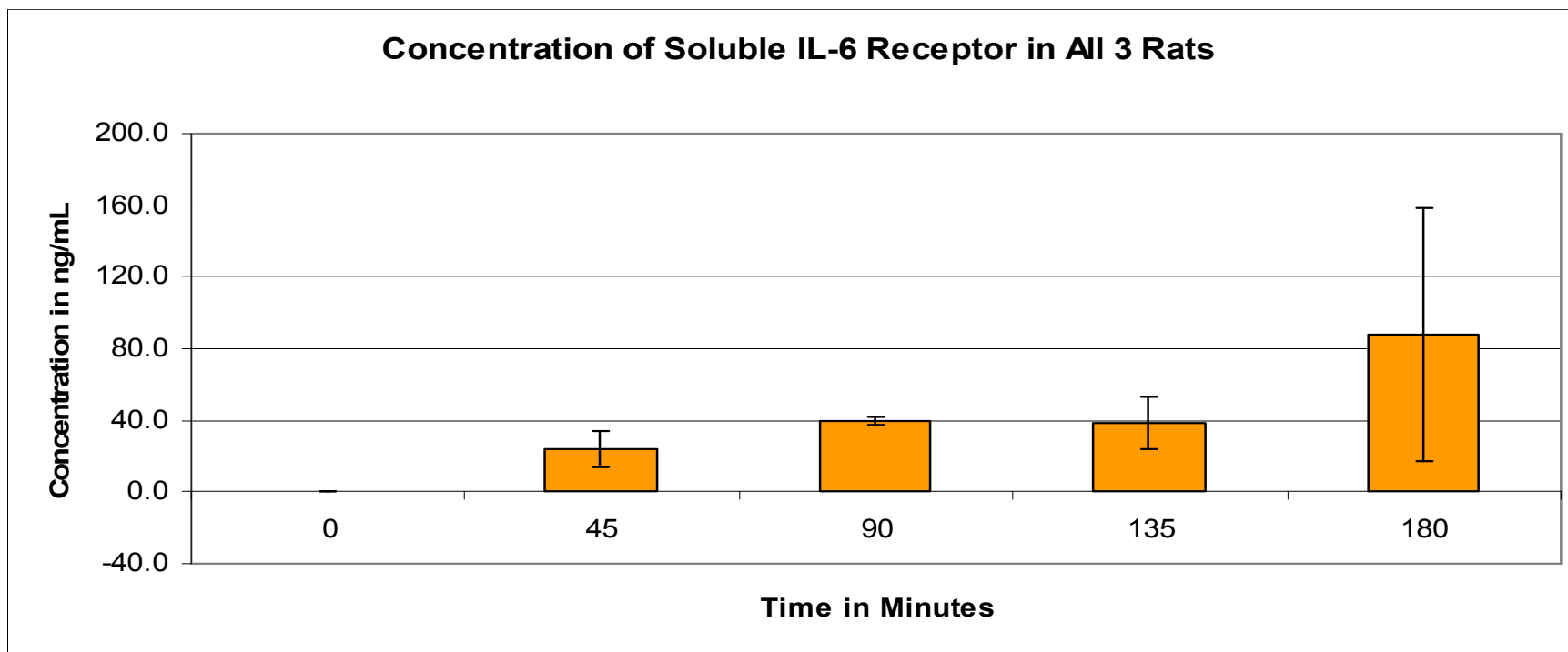
Concentration Soluble IL-6 Receptor - All 3 Rats					
	0	**45**	**90**	**135**	**180**
Mean (ng/mL)	0.0	24.0	39.9	38.7	87.4
Stdev	0.0	10.0	2.1	14.8	70.7
Pearson r Value	0.9377				
Curve P Value	0.0185				

INCREASED APOPTOSIS OF PERIPHERAL BLOOD NEUTROPHILS IS ASSOCIATED WITH REDUCED RISK OF INFECTION IN TRAUMA PATIENTS WITH HEMORRHAGIC SHOCK

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Running Head: Neutrophil Apoptosis and Infection Risk in Trauma

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Abstract:

Introduction: Previous studies have been conflicting with regards to the effects of peripheral leukocyte apoptosis on clinical outcomes in hemorrhagic and septic shock. No studies have examined the correlation between peripheral blood PMN apoptosis and outcomes in trauma patients following resuscitation from hemorrhagic shock *in vivo*.

  Methods: Pre-operative, post-operative, and 24-hour venous samples were drawn from 31 trauma patients requiring emergent laparotomy or thoracotomy. All patients were in hemorrhagic shock and were resuscitated intra-operatively. Leukocyte apoptosis was measured pre-operatively, post-operatively and at 24 hours via nucleosome ELISA and TUNEL staining, and patient records were examined for 30-day mortality, organ failure and infection rates.

  Results: Subjects who survived to hospital discharge without developing any infections had significantly higher post-operative nucleosome levels compared to those who did develop an infection (49.8 mU/mg versus 19.8 mU/mg, p=0.02). This trend persisted when analyzing by specific type of infection. TUNEL staining revealed that 72% of apoptotic cells were PMNs. There were no statistically significant correlations between nucleosome levels and survival.

  Conclusions: In patients with hemorrhagic shock, increased peripheral blood PMN apoptosis is associated with reduced risk of developing subsequent infection. Previous research has shown that high levels of apoptosis in circulating neutrophils following shock may have a protective effect by preventing neutrophil infiltration and limiting release of harmful oxygen radicals in the tissues. Thus, neutrophil apoptosis may render tissues less susceptible to injury and subsequent infection consistent with strategies aimed at benefiting this patient population by limiting PMN number and aberrant function early in the resuscitation period.

Introduction:

Trauma continues to be an enormous public health problem and a leading cause of death around the world.[1] In the United States, trauma is the fifth leading cause of death overall and is the number one cause of death for individuals between the ages of 1 and 45 years of age.[2] With over 170,000 trauma deaths in the US in 2005, more Americans died of injuries than from breast, colon, prostate, liver and pancreatic cancer combined.[3] Historically, the majority of trauma victims died before reaching the hospital[4]; however, more recent epidemiologic studies have shown that advances in trauma systems and emergency medical services (EMS) have resulted in a significantly larger percentage of patients who survive to hospital admission.[5] Although exsanguination and head injury continue to account for a large proportion of early trauma deaths, the vast majority of late deaths occur as a result of infection and/or multiple organ failure (MOF)[5].

The clinical association between late trauma deaths and the development of overwhelming infection and MOF has been well established since the 1970's.[6] However, it has primarily been in the past two decades or so that researchers have focused their investigation into the body's immunological response to trauma/resuscitation on the molecular and cellular level. Leukocyte activation and recruitment to injured tissues have been strongly implicated as causative factors in the development of severe post-traumatic infections in the trauma population.[6] Furthermore, the potential limiting effect of leukocyte apoptosis on initial inflammatory response, tissue injury and subsequent risk of infection in trauma patients is increasingly being studied. To date, the trauma literature has produced somewhat conflicting results with regards to the effects of peripheral leukocyte apoptosis on clinical outcomes, particularly infectious outcomes. Although numerous animal studies have been performed, data from human trauma patients is limited, and much remains to be learned. This paper, to our knowledge, is the first to describe effects of early peripheral blood leukocyte apoptosis on subsequent risk of infection following initial resuscitation in trauma patients in a clinical setting. In keeping with the theory that increased levels of leukocyte apoptosis may reduce inflammatory response and limit local tissue injury and susceptibility to infection following severe trauma and hemorrhage, our hypothesis is that trauma patient with increased levels of leukocyte apoptosis in the early post-resuscitation period will be less likely to develop subsequent infection.

Methods:

This research was conducted at Ben Taub General Hospital, a level-one trauma center located in Houston, TX. Trauma patients arriving to the Emergency Center (EC) with a systolic blood pressure less than 90mmHg who required emergent laparotomy or thoracotomy were enrolled and brought immediately to the operating room (OR) where they underwent simultaneous fluid resuscitation and repair of their injuries. All samples and outcomes data were collected prospectively as part of a separate ongoing clinical trial at our institution comparing hypotensive

resuscitation to standard fluid resuscitation for trauma patients in hemorrhagic shock. Inclusion criteria for the study included traumatic injury to the chest and/or abdomen requiring emergent laparotomy or thoracotomy and at least one documented systolic blood pressure less than 90mmHg. Exclusion criteria included any of the following: 1) patients >45 years or <14 years of age; 2) pregnant women; 3) incarcerated individuals; 4) patients with known history of coronary artery disease, renal disease or cerebrovascular disease; 5) patients in whom traumatic brain injury could not be definitively ruled out based upon mechanism of injury and/or negative CT scan of the head.

Peripheral venous samples were drawn at three time-points: pre-operatively, post-operatively, and at 24 hours. Pre-operative samples were collected either in the EC or in the OR immediately preceding the start of the case; post-operative samples were drawn upon transfer from the OR to the SICU; and 24-hour samples were drawn at 24 hours after admission to the ICU. The timing of the immediate post-operative blood sample was based upon our previous finding that peak levels of apoptosis typically occur within 4 hours of initiating fluid resuscitation following hemorrhagic shock in animals[7-9]. Five mL of blood was collected into heparinized tubes. Peripheral blood leukocytes were then harvested by dextran sedimentation, as described[10], and the pellets immediately frozen for protein extraction. Protein was extracted using lysis buffer (Roche) and quantified using the Bradford method. Nucleosome ELISA was then performed using the Cell Death Detection ELISAplus® kit (Roche). In addition to the nucleosome ELISA assay, cytospins were prepared from leukocytes isolated from 13 of the post-operative blood samples and TUNEL stained as described.[7] The percentage of TUNEL-positive leukocytes, polymorphonuclear leukocytes (PMN) and mononuclear cells within each sample was enumerated microscopically at 1,000x within 20 random fields.

Patients were followed daily for 30 days or until death or hospital discharge. All incidences of infection and/or organ failure were recorded, and these outcomes were analyzed with regards to post-operative peripheral leukocyte apoptosis. Definitions of post-operative complications are shown in Table 1.

Statistical analysis was performed using STATA® statistical software package, version 10.0 (StataCorp, College Station, TX). Comparisons of continuous, independent variables were performed using the Wilcoxon Mann-Whitney test. Comparisons of continuous, paired variables were performed using the Wilcoxon Signed Rank test. Comparisons of categorical variables were performed using chi-squared analysis or Fisher's exact test, as appropriate.

Results:

41 patients had post-operative blood samples drawn and were included in the study. The subjects' baseline characteristics are shown in Table 2. The high prevalence of racial minorities noted in this study roughly reflects the racial breakdown of penetrating trauma victims treated at our urban, county hospital (50% Hispanic, 35% Black, 14% White, 1% Other). Intra-operative fluid requirements are shown in Table 3. The mean time from presentation to the emergency center until arrival in the OR was 15.6 minutes. Mean duration of surgery was 114 minutes. Following surgery, all patients were transferred to the SICU for post-operative care, where post-operative blood samples were drawn. There were no statistically significant differences between those patients who developed an infection and those patients who did not develop any infections with regards to any of the factors listed in Tables 2 and 3(p>0.05 for all comparisons), with the exception of colloid administration. Patients who did not develop infection received less colloid intra-operatively than those who did develop infection (530cc versus 910cc, p=0.01). There was no significant association between risk of developing infection and randomization group for the randomized controlled trial.

For the combined groups, nucleosome levels increased post-operatively compared to pre-operative levels and then dropped to near-baseline levels at 24 hours, as shown in Figure 1. The difference between pre-operative and post-operative nucleosome levels barely failed to reach statistical significance (p=0.06), whereas the difference between post-operative and 24-hour levels was statistically significant (p=0.03).

Of the 41 subjects in whom post-operative samples were obtained, 6 died within 24 hours of surgery. Of the 35 survivors, 17 (48.6%) went on to develop some type of infection over the next 30 days, 3 of whom subsequently died. Eight of the 17 subjects who developed infections had multiple (≥2) infections. 18 subjects who survived past the initial 24 hours never developed any type of infection during the next 30 days. One of these patients subsequently died. The mean number of infections was 1.8 (range 1 to 5). The incidence of each type of infectious complication is listed in Table 4. With regards to each of the characteristics shown in Tables 2 and 3, there were no statistically significant differences at baseline between those who developed an infection versus those who did not develop any infection (p>0.05 for all comparisons).

Subjects who survived to hospital discharge without developing any infections had significantly higher nucleosome levels pre-operatively (40.2 mU/mg versus 13.6mU/mg, p=0.04) and post-operatively (49.8 mU/mg versus 19.8 mU/mg, p=0.02) compared to those who did develop an infection. Twenty-four-hour samples were similar between the two groups. Furthermore, there was a non-significant trend for subjects who did not develop infections to have an increase in nucleosome levels post-operatively, followed by a decrease in nucleosome levels 24 hours later. This same trend was not seen among those who did develop a post-operative infection (Figure 2).

When looking at specific types of infections, subjects who did not develop a given infection consistently had higher pre-operative and post-operative nucleosome levels, although differences

in post-operative levels were more pronounced (Tables 5 and 6). Post-operative nucleosome levels were 2 to 4 times higher in patients who did not develop infections (with the exception of intra-abdominal abscess and wound infection). These differences were found to be statistically significant (p<0.05 for any infection, sepsis, and multiple infections) or nearly significant (p=0.051 and p=0.067 for pneumonia and urinary tract infection, respectively).

TUNEL analysis was performed on a subset of 8 consecutive peripheral blood samples from our study population, in order to determine which type of leukocytes (PMNs versus mononuclear cells) were predominantly undergoing apoptosis. Overall, 9% of all peripheral leukocytes were apoptotic. PMNs accounted for 72% of apoptotic cells. The sample size was too small to make any meaningful comparisons based upon infection status.

Because all subjects were also enrolled in a randomized trial of hypotensive resuscitation at our institution, statistical analysis was performed to identify any significant associations between randomization group and the outcome measures for this study. There was no significant effect of randomization on pre-operative, post-operative, or 24-hour nucleosome levels. There was also no significant association between randomization group and development of any infection versus no infection, nor was there a significant association between randomization group and development of any of the specific types of infection listed in Table 1. Because subjects all received varying amounts of IV fluids and/or transfusions, there was concern that the degree of fluid resuscitation may have a confounding effect on the results. However, statistical analysis revealed no significant differences in the amount of these individual fluids or amount of overall fluids and development of any infection. Neither was the amount of crystalloid, colloid, blood products or total OR fluids significantly associated with development of any infection on logistic regression.

Discussion:

Much of the disagreement in the literature over whether leukocyte apoptosis increases or decreases risk of infection following hemorrhagic shock depends upon which leukocyte populations are being examined. Studies involving lymphocytes have correlated increased levels of apoptosis within primary lymphoid organs such as the thymus and spleen with elevated risk of subsequent infection.[11-14] Presumably the increased infection risk is due to immunosuppression caused directly by depletion of functional lymphocytes within these organs.

On the other hand, several studies of polymorphonuclear leukocytes (PMNs), or neutrophils, have convincingly shown that increased levels of PMN apoptosis may actually reduce the severity of subsequent infection[15,16] and MOF.[17,18] TUNEL staining of peripheral blood leukocyte cytospins in our study demonstrated that 72% of the apoptotic leukocytes detected in the early post-operative period were PMNs. In keeping with these findings, and based upon other results presented in this paper, we propose that increased peripheral blood PMN may protect the severely-injured trauma patient from developing subsequent infection, by one or both of two mechanisms. The first mechanism is through reducing immune-mediated tissue injury; the

second mechanism involves restricting immunosuppression associated with clearance of apoptotic PMNs.

Apoptotic PMNs within the circulation are removed by macrophages within the liver and spleen rather than infiltrate into organs contributing to inflammation and injury. Preventing tissue injury and necrosis in this manner, may reduce the risk for subsequent organ failure as well as the likelihood that the organ will become a subsequent nidus (or source) for infection.

It is well-established that PMNs play an integral role in the body's initial inflammatory response to trauma and are recruited to sites of injury.[6] Once sequestered, these cells possess tremendous potential to induce additional injury to the tissues to which they are recruited. Tissue injury from infiltrating PMNs is not merely limited to organs that have been directly injured from trauma. Ischemia-reperfusion injury (which occurs after resolution of hemorrhagic shock) also results in the recruitment of neutrophils to the ischemic organs. In fact, neutrophil-mediated organ injury following resuscitation from hemorrhage has been demonstrated in liver, heart, kidney, and intestine.[6]

Additionally, activated PMNs stimulate macrophages to release inflammatory cytokines and chemotactic substances which further amplifies the systemic inflammatory response and increases the risk of developing MOF.[19] The presence of large numbers of activated PMNs has been repeatedly associated with development of systemic inflammatory response syndrome (SIRS),[6,17,20] which itself is associated with increased risk of MOF and infection.[17,19] Patients with SIRS/MOF have been shown to have delayed PMN apoptosis[20], enhanced PMN oxidative burst activity,[18,20] and it has been suggested that they have increased sequestration of PMN's as well.[18] It therefore follows that if PMN's could be removed from the circulation before their infiltration in end-organs and without inciting an inflammatory response, then the risk of tissue injury and susceptibility to infection might be reduced. [,16, 17]

An alternative mechanism to explain the finding of reduced infection in trauma patients with increased peripheral blood PMN apoptosis involves apoptosis-mediated immunosuppression. It is well established that apoptosis of PMN within the tissue is accompanied by localized immunosuppression.[21,22] Ingestion of apoptotic cells by macrophages results in the release of anti-inflammatory mediators, including TGF- 1 and PGE_2 and suppresses the production of proinflammatory cytokines such as IL-8 and TNF- , as well as other proinflammatory mediators, including TXA_2, by macrophages. Clearance of apoptotic PMN by the liver and spleen may restrict immunosuppression to these organs thereby sparing other organs such as the lung, which is among the most common sites of infection following serious trauma that leads to MOF and death.

There are several limitations to this study. A major limitation is the relatively small sample size, which makes it difficult to determine significant differences between groups. In several instances, differences just barely failed to reach statistical significance, and it is possible that with a larger sample size, these differences might have been statistically significant. Another

limitation is the presence of numerous potential confounders, which could not be controlled for. In conclusion, this study demonstrates that increased peripheral blood PMN apoptosis in the early post-operative period is associated with decreased risk of developing subsequent infection in severely injured trauma patients requiring emergent laparotomy or thoracotomy. These findings add additional support to the hypothesis that strategies aimed at limiting PMN number and aberrant function early in the resuscitation period may be beneficial in this patient population.

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Table 1: Definitions of post-operative infection.

Patients must exhibit all of the listed features

Pneumonia	
	1. Infiltrate on chest x-ray
	2. Positive sputum culture
	3. Fever and/or leukocytosis
Wound Infection	
	1. Erythema and/or wound drainage
	2. Fever and/or leukocytosis
	3. +/- positive wound culture
Urinary Tract Infection	
	1. Positive urine culture and/or urinalysis
	2. Fever and/or leukocytosis
Intra-abdominal Abscess	
	1. Fluid collection requiring drainage
	2. Fluid described as purulent
	3. +/- positive cultures
Sepsis	
	1. Meets SIRS criteria
	2. Positive blood culture

Table 2: Baseline characteristics of patients (n=41).

Demographics	
Age	32.5 (+/- 9.3)
% Male	92.8%
Race	
Black	51.4%
Hispanic	46.3%
Asian	2.4%
Mechanism	
Blunt trauma	2.4%
Gunshot wound	75.6%
Stab wound	22.0%
Presenting Vital Signs	
Systolic BP	**75** (+/-24)
Diastolic BP	**40** (+/-19)
Pulse	**104** (+/-36)
Baseline Labs	
Base Deficit	**-12.0** (+/- 7.0)
Hemoglobin	**31.1** (+/1 6.2)
Glucose	**214** (+/-84)
Injury Severity	
RTS	**7.1** (1.1)
ISS	**22.0** (+/-12)
TRISS	**0.88** (+/-0.27)

Table 3: Amount and type of fluids administered during intra-operative resuscitation.

IV Fluids	
Crystalloid (mL)	**3450** (+/-1920)
Colloid (mL)	**650** (+/-460)
Transfusions	
PRBC's (mL)	**2100** (+/-2400)
FFP (mL)	**460** (+/800)
Platelets	**100** (+/-210)
Total Transfusions	**2660** (+/-3250)
Total Inputs	**6750** (+/- 4270)

Table 4: Of the 35 patients who survived >24 hours, number and percentage who developed an infectious complication over the next 30 days.

	n	%
Pneumonia	11	31.4
Intra-abdominal Abscess	9	25.7
Sepsis	5	14.3
Urinary Tract Infection	4	11.4
Wound Infection	4	11.4
Empyema	1	2.9

Table 5: Comparison of pre-operative nucleosome levels (mU/mg protein) in peripheral leukocytes, by outcome.

	Disease/Condition Not Present		Disease/Condition Present			
	n	Median Nucleosome Level (mU/mg protein)	n	Median Nucleosome Level (mU/mg protein)	z-score	p-value
Any Infection	13	40.2	17	13.6	2.03	0.04*
Pneumonia	19	26.6	11	6.6	2.11	0.03*
Wound Infection	26	24.2	4	15.1	0.34	0.73
Urinary Tract Infection	26	24.2	4	15.1	0.21	0.83
Abdominal Abscess	21	26.1	9	18.6	0.27	0.79
Sepsis	25	26.6	5	6.6	2.00	0.04*
Multiple Infections	21	26.6	9	6.6	1.36	0.17

*Analysis was performed using the Wilcoxon-Mann-Whitney test; $p < 0.05$ is considered statistically significant.

Table 6: Comparison of post-operative nucleosome levels (mu/mg protein) in peripheral leukocytes, by outcome.

	Disease/Condition Not Present		Disease/Condition Present			
	n	Median Nucleosome Level (mU/mg protein)	n	Median Nucleosome Level (mU/mg protein)	z-score	p-value
Any Infection	18	49.8	17	19.8	2.28	0.02*
Pneumonia	24	46.8	11	19.8	1.95	0.051
Wound Infection	31	39.8	4	27.4	1.30	0.19
Urinary Tract Infection	31	39.8	4	14.2	1.82	0.07
Abdominal Abscess	26	39.0	9	26.1	0.57	0.57
Sepsis	30	42.8	5	10.2	2.17	0.03*
Multiple Infections	26	46.8	9	10.2	2.45	0.01*

*Analysis was performed using the Wilcoxon-Mann-Whitney test; p<0.05 is considered statistically significant.
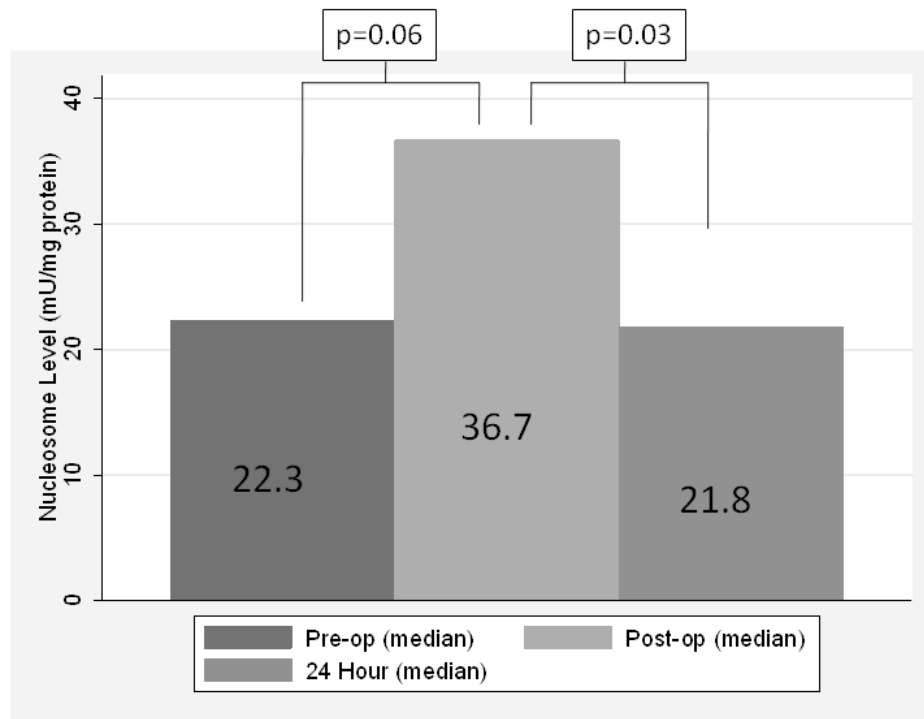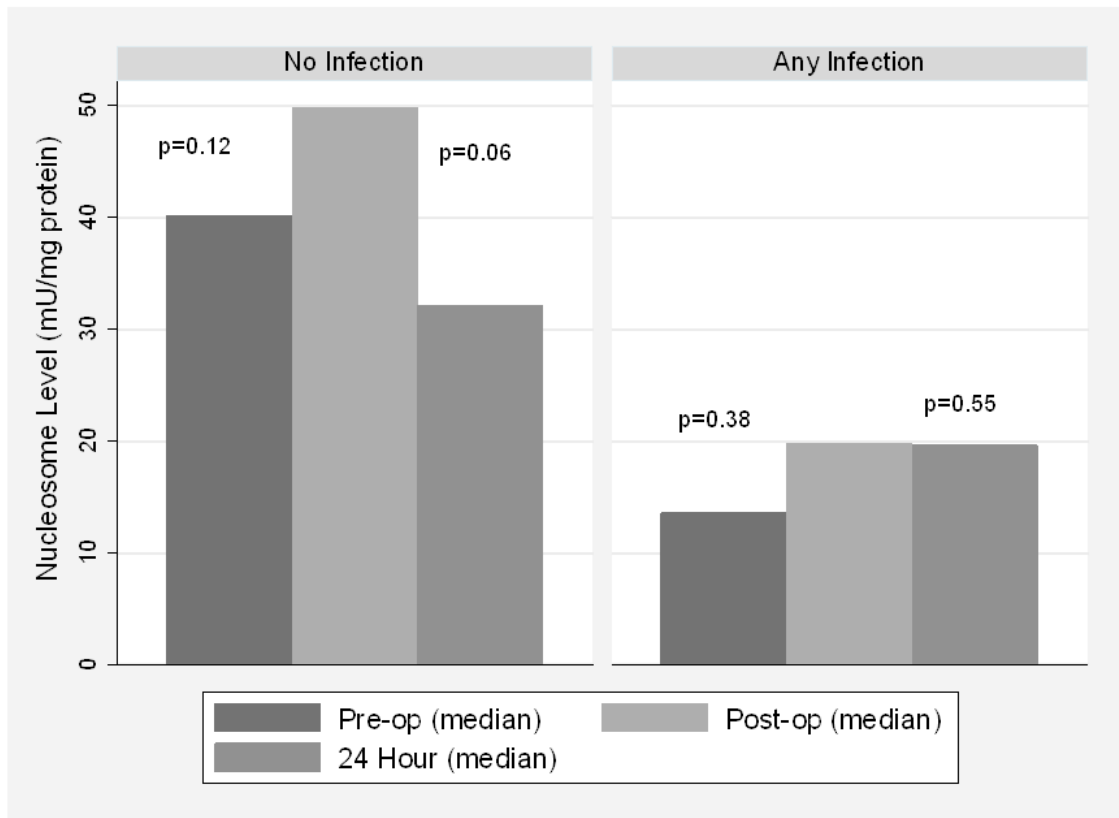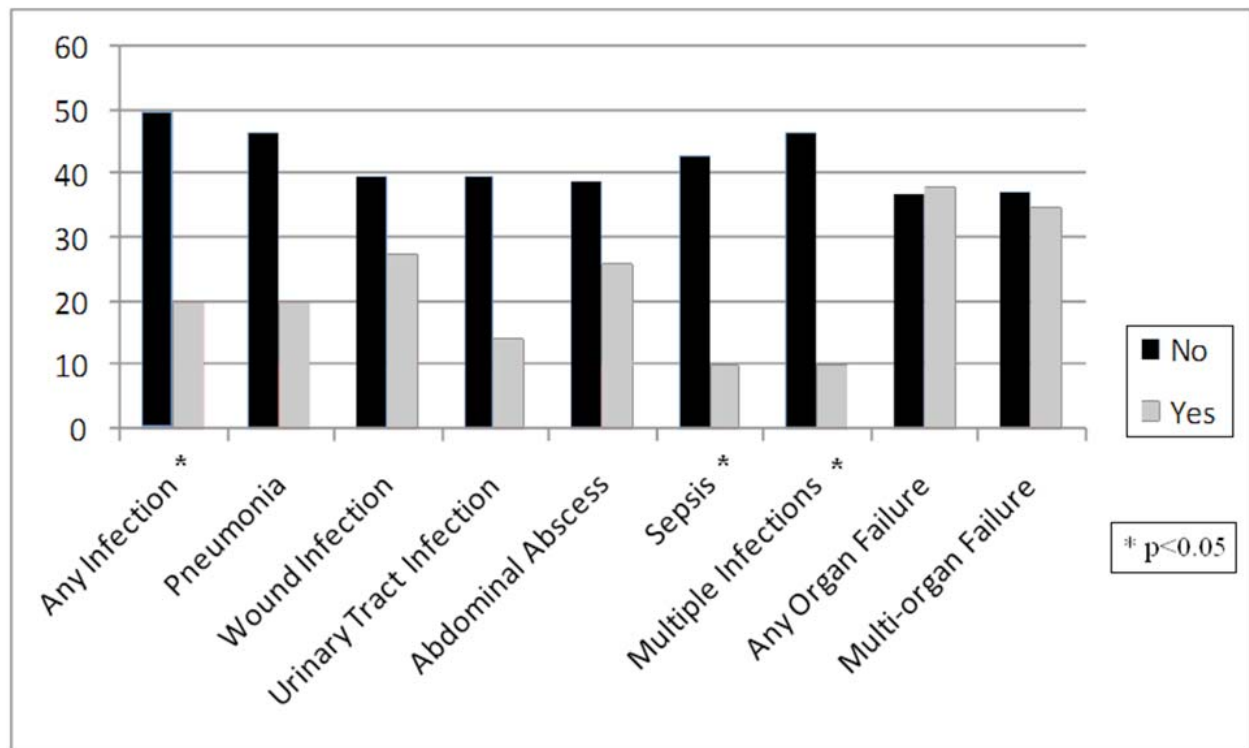
FIGURES

Figure 1

Figure 2

Figure 3

IL-6-Mediated Activation of Stat3α Prevents Trauma/Hemorrhagic Shock-Induced Liver Inflammation

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Abstract

Trauma complicated by hemorrhagic shock (T/HS) is the leading cause of morbidity and mortality in the United States for individuals under the age of 44 years. Initial survivors are susceptible to developing multiple organ failure (MOF), which is thought to be caused, at least in part, by excessive or maladaptive activation of inflammatory pathways. We previously demonstrated in rodents that T/HS results in liver injury that can be prevented by IL-6 administration at the start of resuscitation; however, the contribution of the severity of HS to the extent of liver injury, whether or not resuscitation is required, and the mechanism(s) for the IL-6 protective effect have not been reported. In the experiments described here, we demonstrated that the extent of liver inflammation induced by T/HS depends on the duration of hypotension and requires resuscitation. We established that IL-6 administration at the start of resuscitation is capable of completely reversing liver inflammation and is associated with increased Stat3 activation. Global assessment of the livers showed that the main effect of IL-6 was to normalize the T/HS-induced inflammation transcriptome. Pharmacological inhibition of Stat3 activity within the liver blocked the ability of IL-6 to prevent liver inflammation and to normalize the T/HS-induced liver inflammation transcriptome. Genetic deletion of a Stat3β, a naturally occurring, dominant-negative isoform of the Stat3, attenuated T/HS-induced liver inflammation, confirming a role for Stat3, especially Stat3α, in preventing T/HS-mediated liver inflammation. Thus, T/HS-induced liver inflammation depends on the duration of hypotension and requires resuscitation; IL-6 administration at the start of resuscitation reverses T/HS-induced liver inflammation, through activation of Stat3α, which normalized the T/HS-induced liver inflammation transcriptome.

Introduction

Trauma complicated by hemorrhagic shock (T/HS) is the leading cause of death for those under 45 years old in the United States [1]. Initial survivors of T/HS are particularly susceptible to developing a systemic inflammatory response that triggers multiple organ failure (MOF), a therapeutic challenge and the leading cause of death among these patients [2,3]. MOF is thought to be caused, at least in part, by excessive or maladaptive activation of inflammatory pathways [3-5]. The liver is one of the organs most frequently affected by T/HS, and its central role in metabolism and homeostasis makes this organ a critical one for survival of the host after severe injury [6,7].

We have previously demonstrated that T/HS in a rodent model results in liver injury as evidenced by liver necrosis and inflammation [8], apoptosis [9] and elevated transaminases [10], and that administration of IL-6 at the start of resuscitation prevented liver necrosis and apoptosis [8,9]. However, the contribution of the severity of HS to the extent of liver injury, whether or not resuscitation is required and the mechanism(s) for the IL-6 protective effect have not been explored. In the studies

reported herein, we demonstrated that the extent of liver inflammation induced by T/HS depends on the duration of hypotension and requires resuscitation. We established that IL-6 administration at the start of resuscitation completely prevents liver inflammation and is associated with increased Stat3 activation. Microarray analysis of the livers showed that the main effect of IL-6 was to normalize the T/HS-induced inflammation transcriptome. Pharmacological inhibition of Stat3 activity within the liver blocked the ability of IL-6 to prevent liver inflammation and to normalize the T/HS-induced liver inflammation transcriptome. Genetic deletion of a Stat3β, a naturally occurring, dominant-negative isoform of the Stat3, attenuated T/HS-induced liver inflammation, confirming a role for Stat3, especially Stat3α, in preventing T/HS-mediated liver inflammation.

Methods

Ethics Statement. Animal studies were approved by the Baylor College of Medicine Institutional Review Board for animal experimentation and conform to National Institutes of Health guidelines for the care and use of laboratory animals (Protocol

Approval ID: AN-1980). All animals were sacrificed under general anesthesia as part of our shock protocol to ameliorate suffering.

Rat and mouse protocols for trauma plus hemorrhagic shock. Adult male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Stat3β homozygous-deficient (Stat3β$^{\Delta/\Delta}$) mice were generated as described [11] and re-derived at Jackson labs. Pups from heterozygous matings were tailed and genotyped by PCR, as described, with minor modifications [11].

For the rat experiments in this study, 8-week old male Sprague-Dawley rats (200–250 gm) were used. Rats were subjected to the sham or T/HS protocols, as described [9,12,13] with modifications. Blood was withdrawn into a heparinized syringe episodically to maintain the target MAP at 35 mmHg until blood pressure compensation failed. Blood was then returned as needed to maintain the target MAP. The amount of shed blood returned (SBR) defined 5 different levels of shock severity reflected in the duration of hypotension: 0% SBR (SBR0) represented the lowest level of shock severity (duration of hypotension, 78 ± 2.5 minutes), 10% SBR (SBR10; duration of hypotension, 149 ± 41.4 minutes), 20% SBR (SBR20; duration of hypotension, 165 ± 32.7 minutes), 35% SBR (SBR35; duration of hypotension, 211 ± 7.6 minutes), and 50% SBR (SBR50; duration of hypotension, 273 ± 24.9 minutes). At the end of the hypotensive period, rats were resuscitated as described [9,12,13] and humanely sacrificed 60 minutes after the start of resuscitation in order to capture the first wave of transcriptional changes. Where indicated, rats received 10 µg/kg of recombinant human IL-6 in 0.1 ml PBS at the initiation of the resuscitation or PBS alone. Sham rats were anesthetized and cannulated for 250 minutes but were not subjected to hemorrhage or resuscitation. One group of rats (UHS) was subjected to the most severe hemorrhagic shock protocol (50% SBR), but not resuscitated and kept at the target MAP (35 mmHg) for an additional 60 minutes (duration of hypotension = 336 ± 10.3 minutes) before sacrifice.

In the mice experiments, Stat3β$^{\Delta/\Delta}$ mice and wild-type littermate mice were subjected to a T/HS protocol [9,14], which was similar to the rat protocol except that the target MAP in the mouse was 30 mm Hg and the duration of hypotension was 300 min in all mice. Sham mice were anesthetized and immobilized in a pair-wise fashion with T/HS mice and sacrificed at the same time as their T/HS companion.

Rat and mouse livers were harvested immediately after sacrifice. The right liver lobe was fixed with paraformaldehyde solution (2%) for histological analysis and the left lobe was snap frozen in liquid nitrogen for protein and RNA extraction.

In vivo pharmacological inhibition of Stat3. To achieve pharmacological inhibition of Stat3 activity within the livers, rats were randomized to receive by tail vein injection the G-rich, quartet-forming oligodeoxynucleotide (GQ-ODN) T40214 or non-specific (NS)-ODN (2.5 mg ODN/kg) complexed in polyethyleneimine, as described [15], 24 hours prior to subjecting them to the SBR50 protocol with IL-6 treatment. The half-life of T40214 in tissues is $\geq$48 hr [16].

Myeloperoxidase (MPO) staining. To detect neutrophil (PMN) infiltration, paraffin-embedded liver sections were rehydrated from Xylene to PBS through a series of decreasing concentrations of ethanol, steamed in citrate buffer and the placed on a DAKO autostainer. Polyclonal rabbit MPO antibody-1 (Lab Vision Corporation) was used as instructed by the manufacturer. Slides were counterstained with hematoxylin as described before [14]. MPO positive cells were assessed microscopically by counting MPO positive cells in 20 random ×1000 high power fields by an experienced histologist blinded to the treatment each rat received. Data is presented as number of MPO positive cells per high power field.

Immunoblotting. Levels of Stat3 activation within the livers of rats were assessed by immunoblotting using whole-tissue extracts of liver sections with mouse monoclonal antibodies to Tyr705 phosphorylated (p)Stat3 (Cell Signaling Technology, Inc., Danvers, MA; 1:1000 dilution for each antibody). Briefly, frozen livers were cut by cryotome into 5-micron sections and resuspended in cell lysis buffer for pStat3 detection (Cell Death Detection ELISA^{plus} Kit, Roche Diagnostics, Manheim, Germany). The supernatant was sonicated in ice 3 times, 10 seconds each. Samples were then centrifuged and the supernatant evaluated by Bradford assay for total protein quantification. Protein samples (60ug total protein) were separated by SDS-PAGE and transferred to a PVDF membrane. To detect pStat3, the membrane was incubated overnight with specific mouse monoclonal antibody and subsequently incubated with goat anti-mouse antibody with horseradish peroxidase (HRP) conjugate (Zymed, San Francisco, CA) for 1 hour. ECL agent (Amersham Biosciences, UK) was used for detection. The membrane was then stripped (using RestoreTM Western Blot Stripping Buffer, PIERCE, Rockford, IL) and immuoblotting performed to detect total Stat3 protein using mouse IgG monoclonal antibody to Stat3 (BD Biosciences, Rockville, MD; 1:1000 dilution) as described before [9,12,13]. Detection was done using ECL agent. Densitometry was performed using ImageQuant TL v2005 software (Amersham Biosciences, Buckinghamshire, England). Results are expressed as the ratio of pStat3 (after background signal subtraction) to total Stat3 signal (after background signal subtraction) for each sample.

RNA isolation and microarray hybridization and analysis procedures. Total RNA was isolated from 4–5 micron cryotome sections of liver using TRIzol® Reagent (Invitrogen, Carlsbad, California) single step RNA isolation protocol followed by purification with RNeasy® Mini Kit (QIAGEN, Hilden, Germany) as instructed by the manufacturer. Gene expression profiling was performed with the Affymetrix Rat Array RAE 230A following Affymetrix protocols used within the Baylor College of Medicine Microarray Core Facility.

Microarray Analysis. We used Affymetrics GCOS, dChip and Array Analyzer (Insightful Corporation) software packages for quality assessment and statistical analysis and annotation. Expression estimation and group comparisons were done with Array Analyzer. Low-level analyses included background correction, quartile normalization and expression estimation using GCRMA [17]. One-way analysis of variance (ANOVA) with contrasts [18] was used for group comparisons on all genes and on the list of inflammation-related genes only. P-values were adjusted for multiple comparisons using the Benjamini-Hockberg method [19] with S+Array Analyzer software as described [9,12,13]. The adjusted p-values represent false discovery rates (FDR) and are estimates of the proportion of "significant" genes that are false or spurious "discoveries". We used a FDR = 10% as cut-off. All microarray data are MIAME-compliant, and relevant array data has been uploaded to Gene Expression Omnibus (GEO) with the following series accession number: GSE27978.

Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated where appropriate. Multiple group comparisons of means were done by one-way analysis of variance (ANOVA). Post hoc analysis was done by Student-Newman-Keuls test for 2-group comparisons of means. Correlation between duration of hypotension and MPO positive cells/hpf was done for each individual study animal by Pearson correlation coefficient. Goodness of fit was evaluated by R-square. All statistical analyses were done on SigmaStat 2.03 (SPSS Inc., Chicago, IL).

Results

T/HS-induced liver inflammation depends on the severity of shock and requires resuscitation. To determine the contribution of the severity of shock to liver inflammation, we assessed MPO-positive cells in the livers of rats subjected to increasing duration of shock. The number of MPO-positive cells/hpf increased with the duration of shock (Pearson correlation coefficient 0.869, p<0.0001), with the number of MPO-positive cells/hpf in the SBR50 group (12.8±0.09) increased 3.1 times over sham levels (4.2±0.01; p<0.001; Figure 1A).

To determine the contribution of resuscitation to liver inflammation, we assessed MPO positive cells/hpf in the livers of rats subjected to T/HS without resuscitation (UHS group) and compared these results with those obtained in the sham and the resuscitated SBR50 groups. The number of MPO-positive cells/hpf in the UHS group (5.5±1.3 cells/hpf) was 2.3 times lower than that of the SBR50 group (12.8±0.8 cells/hpf, p<0.01) and similar to that of the sham group (4.2±0.1 cells/hpf; Figures 2A and 2B). Thus, liver inflammation following T/HS depends on the severity of shock and requires resuscitation.

IL-6 administration at the beginning of resuscitation prevents T/HS-induced liver inflammation through a mechanism that involves Stat3α activation. To evaluate the effect of IL-6 administration on T/HS-induced liver inflammation, we measured MPO positive cells in the livers of rats subjected to T/HS with the most severe T/HS protocol (50% SBR) and randomly assigned to receive either PBS (SBR50) or IL-6 (10 μg/kg, SBR50/IL-6) at the beginning of resuscitation. The number of MPO-positive cells/hpf in the SBR50/IL-6 group (6.2±0.4) was 2.1-fold lower than that of the SBR50 group (12.8±0.8 cells/hpf, p<0.01) and similar to that of the sham group (4.2±0.1 cells/hpf; Figures 2A and 2B).
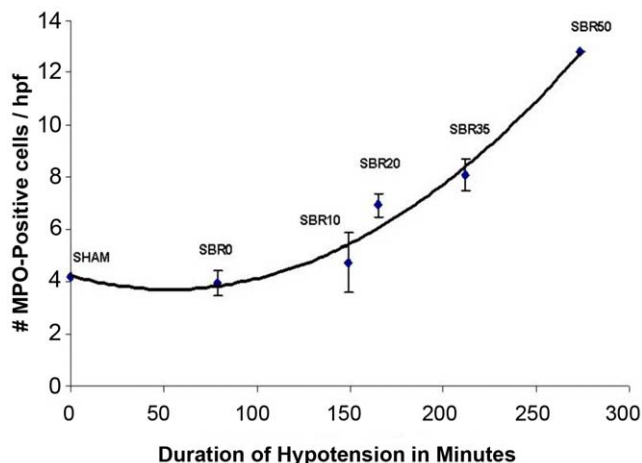

Figure 1. Effect of shock severity on liver inflammation. Rats were subjected to sham protocol (S) or to T/HS protocol with increasing duration of shock as indicated followed by resuscitation. The livers were harvested 60 minutes after the start of resuscitation. Sections of paraformaldehyde-fixed liver were stained to detect myeloperoxidase (MPO) activity and the number of MPO-positive cells counted. Data shown represents the number of MPO-positive cells/high power field in 20 random 1,000x fields. Curve fitting was performed and the best-fitting curve shown; the number of MPO-positive cells increased exponentially with duration of hypotension (Pearson correlation coefficient=0.869, p<0.0001).
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Stat3 is a member of the cytoplasmic protein family that is activated by a large number of extracellular stimuli including IL-6 [20]. Stat3 is known to activate expression of genes responsible for executing anti-inflammatory response, both *in vivo* and *in vitro* [21]. To assess if the anti-inflammatory effect of IL-6 in the liver is mediated by Stat3 activation, we determined if Stat3 is activated in the livers of rats resuscitated with IL-6. Extracts of cryotome sections of the liver harvested 1 hour after IL-6 treatment were examined by immunoblotting with mouse monoclonal antibody to Tyr705 phosphorylated (p)Stat3 (Figure 2C). Densitometric analysis of pStat3 bands normalized for total Stat3 indicated that Stat3 activity in livers of IL-6-treated rats (0.74±0.1 [SD]; n=4) was increased 1.7 fold compared to the livers of placebo-treated rats (0.44±0.08 [SD]; n=4; p<0.05, ANOVA; Figure 2D).

To confirm the role of Stat3 downstream of IL-6 in mediating its anti-inflammatory effect in the liver as well as to assess which isoform of Stat3 mediates it, we examined whether or not these effects of IL-6 could be reversed by pretreatment of rats with a G-rich oligodeoxynucleotide, G-quartet (GQ)-ODN, a novel Stat3 inhibitor, that forms a rigid G-quartet structure within cells, that inhibits the growth of tumors in which Stat3 is constitutively activated [22,23]. Rats were treated in a blinded fashion with GQ-ODN (SBR50/IL-6/G group) or non-specific (NS) ODN (SBR50/IL-6/N group) 24 hours prior to being subjected to T/HS and resuscitation with IL-6 (Figure 2C). Densitometric analysis of pStat3 bands normalized for total Stat3 indicated that Stat3 activity in livers of SBR50/IL-6/G rats (0.26±0.07 [SD]; n=3) was decreased 1.9 fold compared to in the livers of SBR50/IL-6/N rats (0.50±0.09 [SD]; n=3; p<0.05, ANOVA; Figure 2D). Importantly, the inhibition of Stat3 activation within the livers of the SBR50/IL-6/G rats was accompanied by a return of the number of MPO-positive cells (12±0.4 cells/hpf) to levels similar to those of the placebo treated (SBR50) group (12.8±0.8 cells/hpf, p>0.05) and 2-fold higher than those of the IL-6 treated (SBR50/IL-6) group (6.2±0.4 cells/hpf, p<0.01; Figure 2A and 2B). Stat3 activity in livers of SBR50/IL-6/N rats (0.50±0.09 [SD]) was similar to that of the SBR50/IL-6 group (0.74±0.1 [SD]) and was accompanied by a number of MPO-positive cells/hpf that was indistinguishable from that of the SBR50/IL-6 group (Figure 2A and 2B). Thus, pharmacological inhibition of Stat3 using GQ-ODN in rats subjected to T/HS and resuscitated with IL-6 completely blocked IL-6-mediated Stat3 activation and IL-6-mediated prevention of liver inflammation.

Two isoforms of Stat3 are expressed in all cells, α(p92) and β(p83), both derived from a single gene by alternative mRNA splicing with Stat3α predominating. While mice deficient in both isoforms of Stat3 are embryonic lethal at day 6.5 to 7 [24] and mice deficient in Stat3α die within 24 hr of birth, mice deficient in Stat3β have normal survival and fertility [11]. To evaluate the hypothesis that Stat3, in particular Stat3α, contributes to IL-6-mediated prevention of liver inflammation and injury in the setting of T/HS, we subjected Stat3β homozygous-deficient (Stat3β$^{Δ/Δ}$) mice and their littermate control wild type mice to our T/HS protocol (target MAP 30 mm Hg for 5 hr) and examined their livers for MPO-positive cells 1 hr after the start of resuscitation. Similar to our rat model, our mouse model of T/HS induced a 10-fold increase in the number of MPO positive cells/hpf in the wild type mice (16.5±0.79) compared to wild type mice subjected to sham protocol (1.6±0.16; p<0.001; Figure 3). While the number of MPO positive cells/hpf in the Stat3β$^{Δ/Δ}$ mice subjected to T/HS (6.5±0.18) was higher than that of the Stat3β$^{Δ/Δ}$ mice subjected to sham protocol (1.08±0.18; p<0.001), the number of MPO positive cells/hpf in the Stat3β$^{Δ/Δ}$ mice subjected to T/HS was 2.5-fold lower than that of WT mice subjected to T/HS
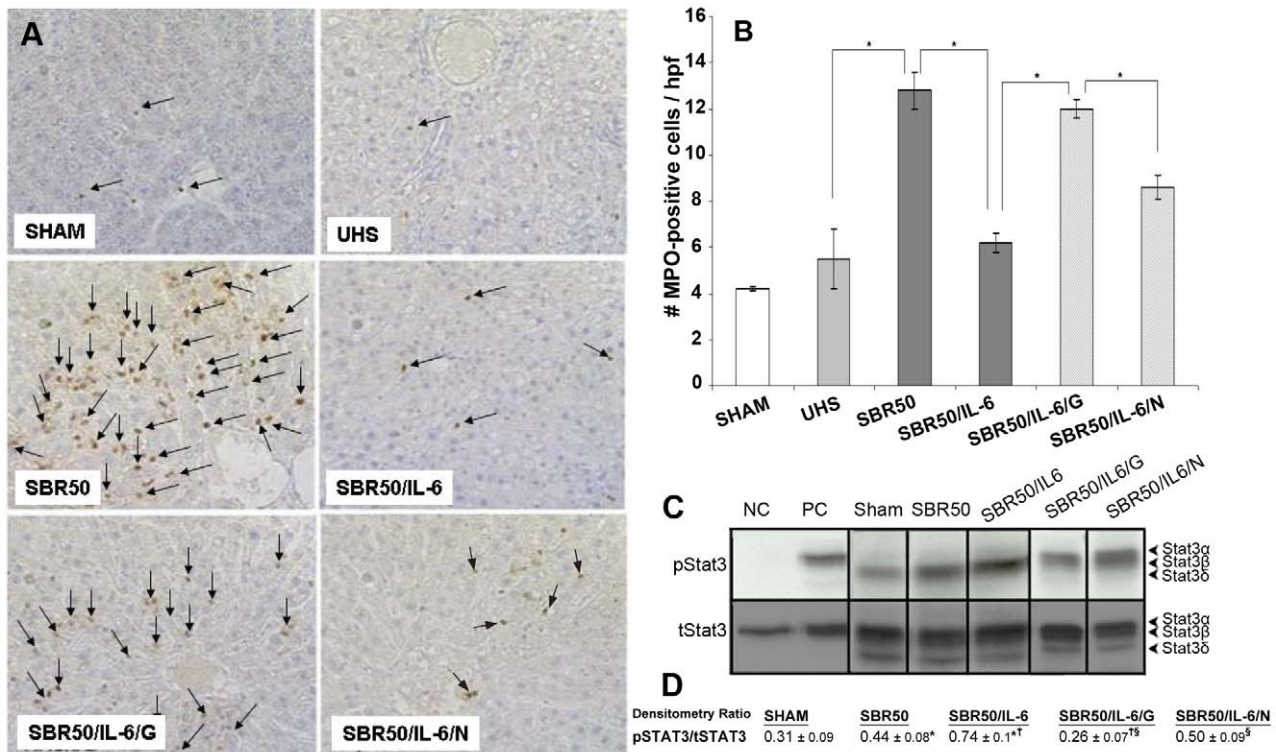
Figure 2. Effect of resuscitation, IL-6 treatment and GQ-ODN pre-treatment on T/HS-induced liver inflammation and Stat3 activation. Rats were subjected to sham protocol (Sham, n = 3), unresuscitated T/HS (UHS, n = 3), T/HS treated with placebo at the beginning of resuscitation (SBR50, n = 4), T/HS treated with IL-6 at the beginning of resuscitation (SBR50/IL-6, n = 4), T/HS preceded by treatment with GQ-oligodeoxynucleotide (GQ-ODN) 24 hours prior to resuscitation with IL-6 (SBR50/IL-6/G, n = 3), or T/HS preceded by treatment with nonspecific-ODN (NS-ODN) 24 hours prior to resuscitation with IL-6 (SBR50/IL-6N, n = 3). The livers were harvested 60 minutes after the start of resuscitation. MPO staining was performed in paraformaldehyde-fixed livers. Representative photomicrographs of 1000x fields of liver specimens from each experimental group are shown (2A). Arrows indicate MPO-positive cells. In Figure 2B, MPO-positive cells were counted in 20 hpf; data presented are the means ± SEM of each group (n≥5). Bars marked with an asterisk (*) differ significantly within the pair (p<0.05). Figure 2C shows representative immunoblots of protein extracts of whole liver from each group, a negative control (NC) cells and positive control (PC) cells developed with monoclonal antibodies to either phosphorylated (p)Stat3 and total (t)Stat3. Bands representing Stat3α, Stat3β, and Stat3δ isoforms are indicated on the right. Heavy lines separate representative samples from individual gels. Figure 2D shows the mean densitometric ratio of (p)Stat3 to (t)Stat3 bands ± standard deviation of the groups (n≥3 for each). Statistically significant (p<0.05) intergroup comparisons are indicated by "*", "†", and "§", respectively.
doi:10.1371/journal.pone.0021449.g002

(p<0.001, Figure 3), indicating that the anti-inflammatory effects of Stat3 in the liver are mediated, at least in part, by Stat3α and that Stat3β inhibits the anti-inflammatory effects of Stat3α.

Microarray analysis of the liver inflammation transcriptome. In the context of normal wound healing and response to injury, Stat3 has been shown to repress transcription of pro-inflammatory genes, thereby promoting protection against necrosis and injury in organ such as the liver and lung [18,25–27]. We have previously shown that IL-6 administration prevents T/HS-induced liver and lung inflammation, in part by blocking NF-κB activation and reducing pro-inflammatory cytokine production [28,29]. To determine the mechanisms involved in this effect as well as to evaluate the role of Stat3 downstream of IL-6, globally, at the transcriptome level within the livers of animals subjected to T/HS especially those genes involved in inflammation in a global and unbiased manner, we performed Affymetrix oligonucleotide microarray analysis with RAE 230A chips. Fifteen chips were hybridized using mRNA isolated from 4 livers each from sham, SBR50, and SBR50/IL-6 groups, and 3 livers from SBR50/IL-6/G groups. All fifteen chips were included in the normalization and expression estimation steps of the analysis and were included in the statistical analysis and differential expression comparison. The

15,866 probesets on the RAE 230A chip represent 9,818 annotated genes or expressed sequence tags, including 694 inflammasome genes. The list of 694 genes belonging to the inflammasome, present on the RAE 230A (Table S1) was created by combining gene lists obtained by querying annotation databases provided in GeneSpring and dChip, which were derived from the Gene Ontology (GO) Consortium.

To identify genes differentially expressed among the experimental groups, the data was filtered to remove genes with nearly uniformly low expression (absent on ≥80% of chips). Of the 694 inflammation transcriptome genes represented on the chips, 583 genes met the requirement of this filtering process and were included in the analysis (Table S1). One-way ANOVA (see Materials and Methods) was then performed which identified 352 inflammation transcriptome genes with differential expression among four experimental groups—sham, SBR50, SBR50/IL-6, and SBR50/IL-6/G—at a False Discovery Rate (FDR) = 10% (Table S1). Of the 352 inflammation transcriptome genes whose expression was altered among the four groups, 235 were altered in the SBR50 vs. sham comparison (Table S2 and Figure 4A). Among the genes whose differential expression was altered in the SBR50 vs. sham comparison, the transcripts of the majority of
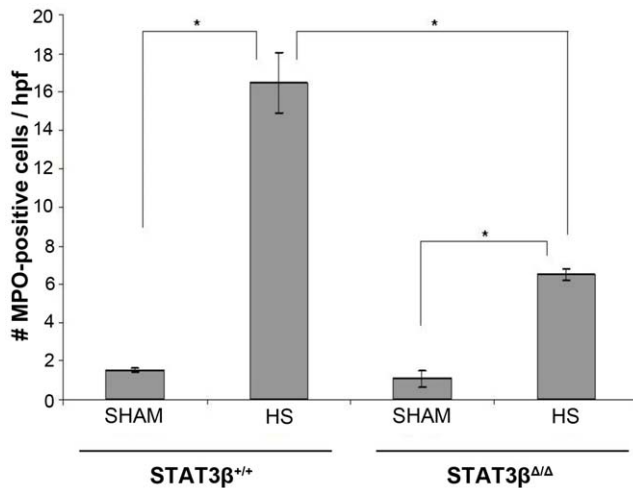
Figure 3. Effect of Stat3β ablation on T/HS-induced liver inflammation. Stat3β homozygous-deficient (Stat3β$^{Δ/Δ}$) mice and their littermate control wild type mice were subjected to the murine T/HS protocol or sham protocol and their livers harvested 1 hr after the start of resuscitation. MPO-positive cells were counted in 20 HPF of paraformaldehyde-fixed liver. Data presented are the means ± SEM of each group (n≥3). Significant differences are indicated (Student's t-test).
doi:10.1371/journal.pone.0021449.g003

these genes (126 genes) were increased in SBR50 vs. sham by 4.05±5.4 fold (range = 1.07 to 45.41 fold) while transcripts of 109 genes were decreased in SBR50 vs. sham by 2.5±1.6 fold (range = 1.1 to 5.3 fold; Figure 4B). Importantly, 104 of the 126 genes that were increased in the SBR50 vs. sham group, were decreased in the SBR50/IL-6 vs. SBR50 group by 1.8±0.9 fold (range = 1.3 to 14.3 fold) and 103 of the 109 genes that were decreased in SBR50 group, were increased significantly in the SBR50/IL-6 group by 1.4±0.7 fold (range = 1.2 to 2.9 fold; Figure 4B). Thus, of the 235 genes whose transcript levels were altered in SBR50 vs. sham group, 207 or 88% returned to sham level or were "normalized" in the SBR50/IL-6 group.

One-hundred and two of the 207 genes, which demonstrated altered expression in the SBR50 vs. sham comparison and normalization in the SBR50/IL-6 vs. SBR50 comparison, also demonstrated altered expression in the SBR50/IL-6 vs. SBR50/IL-6/G comparison. Ninety-seven of these 102 genes (95%) were altered in the opposite direction as the SBR50/IL-6 vs. SBR50 comparison, consistent with the hypothesis that IL-6 normalizes the T/HS-induced liver inflammation transcriptome alterations largely through activation of Stat3.

The inflammation transcriptome contains a list of genes encoding anti-inflammatory proteins that prevent inflammation and genes encoding proteins that induce inflammation. To identify candidate inflammation transcriptome genes whose altered expression mediates T/HS-induced liver inflammation and injury, we focused on both anti-inflammatory genes whose transcript levels were decreased by T/HS and on pro-inflammatory genes whose transcript levels were increased by T/HS. Among the genes differentially expressed in the SBR50 vs. sham comparison, 37 anti-inflammatory genes were decreased and 100 pro-inflammatory genes were increased (Figure 4C). Expression levels of 35 out of 37 (95%) anti-inflammatory genes decreased by T/HS were increased by IL-6 treatment; 84 of 100 (84%) of the pro-inflammatory genes that were increased by T/HS, were decreased by IL-6 treatment. Finally, the expression of 37% of anti-

inflammatory genes increased by IL-6 treatment was decreased by pre-treatment with T40214; conversely 70% of pro-inflammatory genes decreased by IL-6 treatment were increased by T40214 pretreatment (Figure 4C).

Pro-inflammatory genes whose expression was increased≥6-fold by T/HS were *Dual specificity phosphatase 6 (Dusp6*; 16.8-fold), *fatty acid binding protein 4 (Fabp4;* 12-fold), *alpha-2 macroglobulin (A2m,* 10.1-fold), *interferon regulatory factor 1 (Irf1*; 9.3-fold), *fatty acid binding protein 5 (Fabp5;* 9.3-fold), *oxidized low density lipoprotein receptor 1 (Oldlr1*; 9.0-fold), *Ephrin A1 (Efna1;* 7.7-fold), *Phosphatidyl-inositol-3 kinase regulatory subunit polypeptide 1 (Pik3r1;* 6.2-fold) and *BCL2 adenovirus E1B interacting protein 3 (Bnip3*; 6.1-fold, Table 1). The expression of each was decreased in the IL-6-treated group by 1.8- to 7.3-fold (Table S2). The effect of T/HS in the anti-inflammatory subset of genes was more modest. Indeed, none of the genes in this subset was altered more than 5.1-fold.

Discussion

The findings in our rodent protocol of T/HS demonstrated that the extent of T/HS-induced liver inflammation following T/HS depends on the duration of hypotension and requires resuscitation. In previous studies, we had shown that liver inflammation induced by T/HS is associated with liver dysfunction and 72% mortality [12,29], and IL-6 administration at the start of resuscitation completely reversed T/HS-induced liver inflammation and injury, decreasing mortality 5-fold, to 15% [12,29]. In the current studies, we established that IL-6 administration at the start of resuscitation is capable of completely reversing liver inflammation as early as one hour after resuscitation, and is associated with increased liver Stat3 activation. Moreover, pharmacological inhibition of Stat3 using G-quartet oligodeoxynucleotide (GQ-ODN) T40214 developed by our group completely blocked IL-6-mediated liver Stat3 activation and prevention of inflammation. In our model of T/HS, Stat3α was the Stat3 isoform responsible for the anti-inflammatory effect of Stat3 in the liver. Liver microarray analysis showed that 65% of known inflammation transcriptome genes were altered in T/HS. Administration of IL-6 "normalized" the expression of 88% of these genes by increasing the expression levels of 95% of the anti-inflammatory gene transcripts whose levels were decreased by T/HS and by decreasing transcript levels of 84% of the pro-inflammatory genes whose levels were increased by T/HS. Furthermore, in 55% of the cases, Stat3 mediated the normalizing effect of IL-6 administration in the inflammation transcriptome.

The liver is readily susceptible to injury following insults such as hemorrhagic shock. Since the liver is responsible for maintaining homeostasis and is a key source of energy to other organs, hepatic injury and dysfunction associated with hemorrhagic shock can affect other organs and lead to multiple organ failure and death [30–32]. Liver inflammation and injury has been observed by us and others following T/HS [10,29,33–35], which we previously demonstrated can be prevented by administration of IL-6 at the start of resuscitation [28,29]. However, the effect of the duration of hypotension on the development of T/HS-induced liver inflammation, as well as the mechanism(s) mediating the protective role of IL-6 administration have not been reported.

We have previously demonstrated that T/HS induces liver necrosis and polymorphonuclear (PMN) infiltration, which were observed 4 hrs after resuscitation [8,29]. In the current study, using our rodent model of T/HS we found that liver inflammation occurs as early as 1 hour after reperfusion, and for the first time, that its severity depends on the duration of hypotension and requires resuscitation. Liver inflammation and injury have been
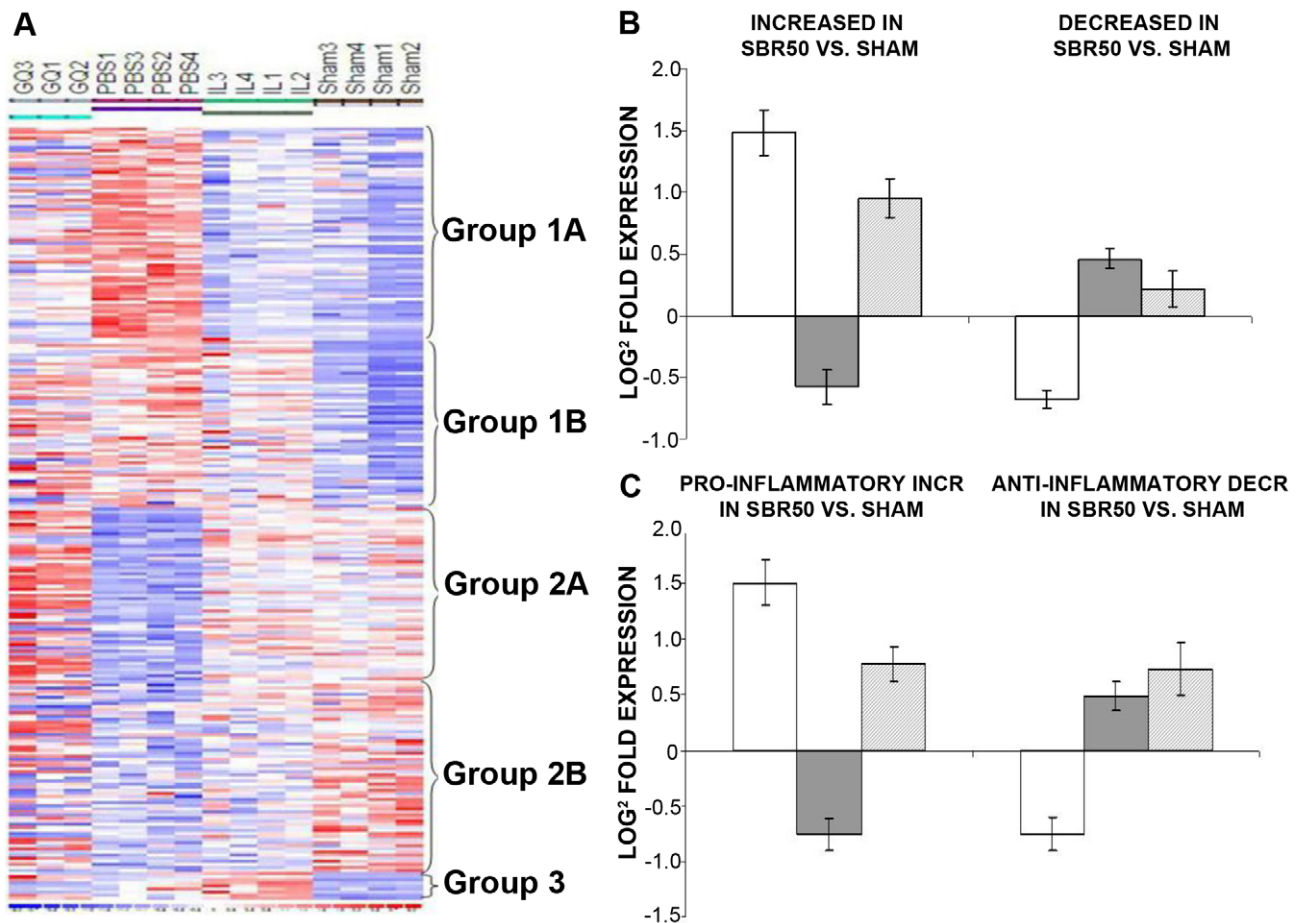
Figure 4. Effect of T/HS without or with IL-6 treatment on the liver inflammation transcriptome; impact of Stat3 inhibition on the IL-6 effect. In Figure 4A, a heat map of the liver inflammation transcriptome is shown containing those genes whose expression is altered within the 4 groups. Columns represent samples from the 4 groups examined as indicated (Sham; PBS, placebo-treated SBR50; IL, IL-6-treated SBR50/IL-6; and GQ, animals pre-treated with G-quartet ODN prior to HS and IL-6 treatment, SBR50/IL-6/G). Rows represent genes as listed in Table S2. Red indicates a level of expression above the mean expression of a gene within the experimental group. White indicates a level of expression at the mean within the experimental group while blue indicates a level of expression below the mean within the experimental groups. In Figure 4B, the 235 inflammation transcriptome genes whose expression levels were changed in SBR50 vs. sham were separated into those genes whose transcript levels were increased in SBR50 vs. sham (126 genes; left side of panel) and those whose transcript levels were decreased in SBR50 vs. sham (109 genes; right side of the panel). Bars shown represent mean $\pm$ SD of the Log_2-fold change in gene expression levels for each comparison. In Figure 4C, the overall effect of T/HS in transcript levels of pro- and anti-inflammatory genes is shown. In the left side of the panel, the mean $\pm$ SD of the Log_2-fold change in gene expression levels of 100 proinflammatory genes whose expression was increased in the SBR50 vs. sham comparison is shown (open bar). The expression of 84 of 100 of these genes was decreased in the SBR50/IL-6 vs. SBR50 comparison (gray bar). In the right side of the panel, the mean $\pm$ SD of the Log_2-fold change in gene expression levels of 37 anti-inflammatory genes whose expression was decreased in the SBR50 vs. sham comparison is shown (open bar). The expression of 35 of these genes was increased in the SBR50/IL-6 vs. SBR50 comparison (gray bar).
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shown to occur in settings of reperfusion injury such as liver transplantation [36,37]. Upregulation of NF-κB target genes, which induce an inflammatory cascade, has been documented to occur early after hemorrhagic shock [28,38]. Furthermore, NF-κB transcriptional activity has been shown to persist long after the initial insult, leading to a sustained inflammatory process, associated with cell injury [31]. Concordant with these data, a possible mechanism, mediating liver inflammation and its correlation with the severity of shock in our model of T/HS, invokes activation of NF-κB, which has been shown to be rapidly activated during reperfusion in hemorrhagic shock and whose binding activity depends on the severity of shock [39]. Activation of NF-κB can, in turn, upregulate the expression of cytokine genes that would promote inflammation [28,29,33]. The presence of nitric oxide, which has been implicated in the development of cell and organ damage in settings of regional hypoxia and low flow,

both relevant to hemorrhagic shock [7], could also contribute to our findings of increased liver inflammation. Indeed, in previous studies, we demonstrated a nitric oxide-dependent increase of proinflammatory cytokines after HS [14,33]. Reactive oxygen species (ROS) generated during reperfusion may also play a role in liver inflammation, as suggested by other investigators, given the ability of ROS to upregulate adhesion molecules and chemotactic factors which perpetuate the inflammatory response [7].

IL-6 is a pleiotropic cytokine, which has been shown to provide liver protection in various settings [40-43]. IL-6-mediated activation of Stat3 has been shown to down-regulate Stat1 activity and to prevent inflammation by blocking interferon-γ-like response *in vitro* [44]. Additionally, we have previously shown that exogenous IL-6 protected against liver PMN infiltration during hemorrhagic shock and was associated with decreased NF-κB activity in the liver 4 hrs after reperfusion [29]. Consistent with

Table 1. Pro-inflammatory genes whose expression was increased ≥6-fold by T/HS.

Pro-inflammatory Gene Name	Symbol	Fold Change
dual specificity phosphatase 6	Dusp6	16.8
fatty acid binding protein 4	Fabp4	12
alpha-2 macroglobulin	A2m	10.1
interferon regulatory factor 1	Irf1	9.3
fatty acid binding protein 5	Fabp5	9.3
oxidized low density lipoprotein receptor 1	Oldlr1	9
Ephrin A	Efna1	7.7
Phosphatidyl-inositol-3 kinase regulatory subunit polypeptide 1	Pik3r1	6.2
BCL2 adenovirus E1B interacting protein 3	Bnip3	6.1

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these findings, in the current study, we demonstrated that IL-6 prevented PMN infiltration as early as 1 hr after reperfusion. Likely explanations for our findings are the down-modulatory effect of IL-6 on NF-κB activity, either directly or as a result of reduced TNF-α or IL-1 production [29] and down-regulation of proinflammatory mediators by IL-6, which has been documented in T/HS animals treated with exogenous IL-6 infusion [28].

The role of Stat3 activation downstream of IL-6 in the resistance to T/HS-induced liver inflammation has not been studied previously. In our rodent model of T/HS we found increased Stat3 activation in the livers of rats subjected to T/HS and treated with IL-6 at the beginning of resuscitation. Furthermore, pharmacological inhibition of Stat3 with GQ-ODN, 40214, decreased liver Stat3 activation and completely prevented the anti-inflammatory effect of IL-6. Thus, IL-6-mediated activation of Stat3 is critical for protection against liver cell injury due to T/HS-induced inflammation.

Several studies have demonstrated that the protective role of Stat3 against liver injury is mediated, in part, by prevention of inflammation. Using a model of T-cell-mediated hepatitis in IL-6$^{-/-}$ mice, Hong et al. showed that IL-6-mediated Stat3 activity at the whole animal level protected the liver against inflammation [45]. Other groups, using a model of partial hepatectomy, have previously demonstrated that decreased activation of IL-6/Stat3 in the isolated hepatocytes of ethanol-fed rats was associated with increased liver inflammation and injury [46]. Furthermore, IL-6-induced activation of Stat3, assessed in total liver nuclear protein extract, has been shown to mediate liver protection against inflammation in a model of concavalin A liver injury using IL-6$^{-/-}$ mice [47].

Similar to bowel inflammation [48–51], Stat3′s contribution to liver inflammation is influenced in a cell-type specific fashion that may functionally compete, depending on the cell type and the model of liver injury. Lafdil et al. recently demonstrated, in a model of T cell hepatitis, that deletion of Stat3 in myeloid cells enhances inflammation while deletion of Stat3 in T cells reduces liver inflammation [52]. Conditional deletion of Stat3 in hepatocytes has been shown to reduce liver inflammation while conditional deletion of Stat3 in myeloid cells enhanced liver inflammation after CCl4 injection [53]. We have previously demonstrated that the organ-sparing effect of IL-6 administration in our T/HS model is mediated by Stat3 activation within parenchymal cells, i.e. hepatocytes, cardiomyocytes, and alveolar

epithelial cells of the liver, hear, and lung, respectively. While the anti-inflammatory effects of IL-6-activated Stat3 in our T/HS model may be mediated by Stat3 activation within myeloid cells, it is not likely to be mediated by Stat3 in T cells, and is most likely to be mediated by Stat3 activated within hepatocytes. However, future studies using the appropriate cell-specific Stat3 knockout animals will be necessary to distinguish between these alternatives.

Stat3 has multiple functions including modulation of target genes, which is in part due to the existence of two distinct isoforms, α (p92) and β (p83), derived from a single gene by alternative mRNA splicing with Stat3α predominating [54]. For example, Stat3α is essential for induction of inflammatory genes in the liver yet it also mediates the anti-inflammatory functions of IL-10 in macrophages [11]. In contrast, Stat3β$^{Δ/Δ}$ mice are more susceptible to organ damage (particularly liver) and mortality due to lipopolysaccharide injection [11,55]. Our finding of decreased MPO-positive cells in the livers of Stat3β$^{Δ/Δ}$ mice subjected to T/HS shock in comparison to their wild type littermates, suggest that Stat3α, and not Stat3β, is the isoform mediating the anti-inflammatory effect of Stat3. The decrease in inflammation is not mediated through differential levels of phosphorylated Stat3 within the isoform-depleted mice, but rather through the activation of distinct transcriptomes [11].

Similar to the prior discussion of Stat3′s varied role in inflammation depending on cell type, the role of Stat3α or Stat3β in inflammation is likely dependent on the target cell type as well as the pro-inflammatory stimulus. This is illustrated by the fact that Stat3α, not Stat3β, is known to mediate anti-inflammatory functions of IL-10 in LPS-stimulated macrophages [11,56]. Additionally, Yu et al. found that in LPS-stimulated mesangial cells, Stat3α inhibited transcriptional activation of the inducible nitric oxide synthase (iNOS) gene by physically and functionally interacting with NF-κB [57]. Of note, we have previously shown that T/HS-induced inflammation and liver injury are mediated, in part, by iNOS transcription by NF-κB [58,59].

Global and unbiased assessment of the liver inflammation transcriptome using oligonucleotide microarray analysis determined that T/HS altered the expression of 65% of inflammation transcriptome genes, of which 73% were pro-inflammatory and 27% were anti-inflammatory genes (S2, Figure 4B). T/HS increased the levels of 58% of proinflammatory genes and decreased the levels of 59% of anti-inflammatory genes. IL-6 administration prevented the T/HS-mediated changes by decreasing the expression of inflammation transcriptome genes whose expression was increased by T/HS, and by increasing the expression of inflammation transcriptome genes whose expression was decreased by T/HS (Figure 4C), indicating that IL-6 administration had a "normalizing" effect on the T/HS-induced liver inflammation transcriptome. Inhibition of Stat3 using GQ-ODN T40214 reversed the IL-6 "normalizing" effect on gene expression in 55% of the inflammation transcriptome genes (Figure 4C). These results suggest that IL-6 administration prevents T/HS-induced liver inflammation by opposing the effects of T/HS on the liver inflammation transcriptome, in part, by Stat3 activation.

Anti- and proinflammatory subsets of genes were analyzed separately to determine the effect of T/HS in each subset of transcripts. Gene transcript levels of the majority of proinflammatory were increased by T/HS. Interestingly, IL-6 normalized the expression of 84% of these genes, an effect that was reversed by pre-treatment with Stat3 inhibitor in the majority of them (66%, Table S2, Figure 4C). In our model of T/HS, proinflammatory genes upregulated by T/HS are likely to be mediators of liver inflammation, which is likely prevented by IL-6 normalizing effect

on their expression through Stat3 activation. Among the transcripts most upregulated following T/HS and downregulated in the IL-6 treatment group were *Dusp6*, *Fabp4*, *A2m*, *Irf1*, *Fabp5*, *Oldlr1*, *Efna1*, *Pik3r1*, and *Bnip3*. Dusp6 is a cytosolic phosphatase with specifically inactivates extracellular signal-regulated kinase (ERK) [60]. *Oldlr1* is expressed in endothelial cells and activated macrophages. Oldlr1 is a type II glycoprotein and acts as a receptor for oxidized low-density lipoprotein (ox-LDL). Interaction with ox-LDL induces ROS, reduces NO and activates NF-κB. *Old1r*1 is known to induce endothelial cell injury by facilitating inflammatory cell adhesion in an animal model of myocardial ischemia-reperfusion [61,62]. *Fabp2* and *Fabp5* have been implicated in the pathogenesis of allergic airway inflammation as well as chronic inflammation associated with cardiovascular disease [63,64]. *A2m* is a major acute phase protein with protease-inhibitory activity, and mice deficient in *A2m* were protected against lethal systemic inflammation induced by TNF [65]. Activation of *Irf1*-dependent autocrine loop is known to induce persistent inflammatory response in an animal model of delayed inflammation [66].

Mediation of the protective effects of IL-6 in T/HS-induced liver injury is in large part through Stat3′s ability to prevent liver inflammation. Our findings provide evidence that support the use of IL-6 as a potential therapeutic agent to protect against liver injury and dysfunction by blocking inflammation early after reperfusion. Such an intervention may prevent multiple organ failure and improve survival in the setting of trauma complicated by severe hemorrhagic shock.

Supporting Information

Table S1 Inflammation transcriptome genes examined in the microarray experiments. Table S1 identifies the members of the inflammasome present on the RAE 230A chip after filtering for uniformly low expression across chips and describes the 352 identified as having differential expression among four experimental groups—Sham, SBR50, SBR50/IL-6, and SBR50/IL-6/G—at a False Discovery Rate (FDR) = 10% via Oneway ANOVA (see Methods section). * "Y" indicates signal detected above background for gene probeset in 20% or more of the chips. † "Y" indicates significant differential gene expression

within the Sham, SBR50, SBR50/IL-6, and SBR50/IL-6/G groups using False Discovery Rate (FDR) = 10%. "N" indicates not significant differential gene expression. "NA" indicates genes not included in the analysis because of not being detected in at least 20% of the chips.
(DOC)

Table S2 Inflammation transcriptome genes differentially expressed in the SBR50 vs. SHAM comparison. Table S2 identifies the 235 members of the inflammasome whose expression was altered among the experimental groups and describes the pattern of dysregulation between and among experimental groups in comparison to the SBR50 vs. Sham animals. Genes are grouped based on direction of dysregulation, allowing for identification of inflammasome genes dysregulated by our trauma/hemorrhagic shock model that are "normalized" by IL-6 and identification of inflammasome genes whose altered expression is, in part, regulated through Stat3. Group 1A represents genes increased in SBR50 vs. Sham and decreased in SBR50/IL-6 vs. SBR50. Group 1B represents genes increased in SBR50 vs. Sham and unchanged in SBR50/IL-6 vs. SBR50. Group 2A presents genes decreased in SBR50 vs. Sham and increased in SBR50/IL-6 vs. SBR50. Group 2B presents genes decreased in SBR50 vs. Sham and unchanged in SBR50/IL-6 vs. SBR50. Group 3 represents genes increased in SBR50 vs. Sham and increased in SBR50/IL-6 vs. SBR50. *Genes listed in regular type are anti-inflammatory, while genes listed in italics are pro-inflammatory. †FDR: False discovery rate.
(DOC)

Author Contributions

Conceived and designed the experiments: AM DJT. Performed the experiments: AM AAA M-AAM YW BY. Analyzed the data: AM SAT DJT. Contributed reagents/materials/analysis tools: DJT. Wrote the paper: AM SAT DJT. Created Figures: SAT AM.

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CONTRIBUTION OF THE UNFOLDED PROTEIN RESPONSE TO HEPATOCYTE AND CARDIOMYOCYTE APOPTOSIS AND ITS PREVENTION IN TRAUMA/HEMORRHAGIC SHOCK

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ABSTRACT

Trauma is a major cause of mortality in the United States. Mortality among survivors of the initial trauma is caused by multiple organ failure with the liver and heart most often affected. Trauma with hemorrhagic shock (T/HS) has been shown to result in liver injury marked by hepatocyte apoptosis and heart failure marked by cardiomyocyte apoptosis. T/HS-induced hepatocyte and cardiomyocyte apoptosis has been shown to be prevented by IL-6 administration at resuscitation, and Stat3 largely mediated this. Specific genes contributing to apoptosis and its prevention; however, were not clearly delineated. Endoplasmic reticulum stress elicits the unfolded protein response (UPR), which, with marked activation, can lead to apoptosis. Prior studies of hepatic and cardiac injury examined a limited repertoire of UPR elements, making it difficult to assess the maladaptive or inadequately adaptive role of the UPR in T/HS. This study describes the first global examination of the UPR transcriptome in the liver and heart in the setting of T/HS, demonstrating the liver and heart exhibit distinct canonical and non-canonical UPR transcriptomes. The non-canonical UPR chaperone, Hsp70, was most dysregulated following T/HS. In the liver, Hsp70 may contribute to hepatocyte protection via a novel IL-6-mediated Stat3-dependent pathway, identifying a potential novel therapeutic strategy to prevent hepatocyte death and organ dysfunction in T/HS.

Keywords: unfolded protein response, hemorrhagic shock, heat shock protein, apoptosis

INTRODUCTION

Trauma is a leading cause of morbidity and mortality in the United States for those under the age of 45 years, especially when complicated by hemorrhagic shock [1]. When trauma with hemorrhagic shock (T/HS) is accompanied with resuscitation, the end effect is essentially a systemic ischemia and reperfusion injury. Multiple organ failure is an important maladaptive sequelae contributing to late mortality in those who survive beyond 24 hrs following severe T/HS and resuscitation [2].

Work done by our group, and others, in rodent models of T/HS, has shown that parenchymal cells within organs such as the liver, a key metabolic and homeostatic organ, and heart, an organ whose dysfunction often heralds post-traumatic mortality, undergo apoptosis [3-7]. The pathways leading to parenchymal cell apoptosis in these organs in T/HS are not fully understood. The classical mechanisms of apoptosis, such as the extrinsic and intrinsic apoptotic pathways, have been investigated in the liver and heart [3, 6]. However, specific delineation of the pathways leading from T/HS to cell death and organ dysfunction is incomplete.

Prolonged or severe endoplasmic reticulum (ER) stress has recently been demonstrated to lead to apoptosis through the unfolded protein response (UPR). The canonical genes involved in ER stress and the UPR were first delineated in yeast including identification of the ER membrane bound sensors of ER stress [8-11]. Homologues for these sensors and their targets have been identified in mammals and

their activation can reliably be assessed transcriptionally. While much of the focus of investigation on the UPR has centered around the three main signaling molecules inositol-requiring enzyme 1α (IRE1α), Activating Transcription Factor 4 (ATF4), and protein kinase RNA-like endoplasmic reticulum kinase (PERK), many non-canonical modulators of the UPR have been identified linking the UPR to pathways ranging from innate immunity to apoptosis. Emerging evidence has shown that prolonged ER stress and UPR activation leads to apoptosis that is an important mechanism of disease pathogenesis in a number of genetic disorders, such as lysosomal storage diseases, particularly within the liver [12, 13]. Examination of the UPR as a potential cause of parenchymal cell apoptosis in metabolic and other derangements leading to ER stress initially focused on exocrine organs such as the liver [14]. The UPR and its contribution to liver disease has been investigated in liver diseases such as steatosis [15, 16], ischemia/reperfusion injury [17, 18] and T/HS [19, 20]. The impact of the ER stress and the UPR on non-exocrine organs such as the heart, has only recently become a focus [21, 22]. Studies of both the liver and heart are limited, however, since they have focused on isolated components of the UPR and did not provide direct evidence that would allow one to conclude that apoptosis or organ injury resulted from an insufficient adaptive UPR or that the UPR or components therein were, in fact, maladaptive.

We previously demonstrated that parenchymal cell apoptosis following T/HS in both the liver and heart is prevented by administration of IL-6, which mediates its effect through the actions of Stat3 [3, 6]. In the current studies, we performed UPR transcriptome analysis of the liver and heart at a global level to identify candidate genes within the

canonical and non-canonical UPR that contribute to apoptosis following T/HS. By tracking the direction and magnitude of changes in levels of these candidate genes that occurred following T/HS with IL-6 resuscitation, with or without Stat3 inhibition, we were able to clearly identify those genes most implicated in T/HS-induced apoptosis and its prevention by IL-6-activated Stat3. In particular, we demonstrated that Hsp70 and 40 were upregulated in the liver by T/HS, and that this response was adaptive and insufficient since IL-6 augmented it, thereby preventing apoptosis, an effect mediated by Stat3.

METHODS

Rat T/HS protocol. For the rat experiments in this study, 8-week old male Sprague-Dawley rats (200-250 gm) were used. Rats were subjected to the sham or T/HS protocols, as described [3, 6, 23] with modifications. Blood was withdrawn into a heparinized syringe to achieve then maintain the target MAP at 35 mmHg until blood pressure compensation failed. Blood was then returned as needed to maintain the target MAP. The amount of shed blood returned (SBR) defined shock severity as reflected in the duration of hypotension, and the animals used in this analysis received 50% SBR (SBR50; duration of hypotension, 273 ± 24.9 minutes). At the end of the hypotensive period, rats were resuscitated as described [3, 6, 23] and humanely sacrificed 60 minutes after the start of resuscitation in order to capture the first wave of transcriptional changes. Where indicated, rats received 10 µg/kg of recombinant human IL-6 in 0.1 ml PBS at the initiation of the resuscitation or PBS alone. Sham rats were anesthetized and cannulated for 250 minutes but were not subjected to hemorrhage or resuscitation. Rat livers and hearts were harvested immediately after sacrifice and snap frozen in liquid nitrogen for protein and RNA extraction steps.

In vivo pharmacological inhibition of Stat3. To achieve pharmacological inhibition of Stat3 activity within the rats, the G-rich, quartet-forming oligodeoxynucleotide, T40214[24] (GQ), or nonspecific ODN (2.5 mg ODN/kg) was given by tail vein injection, 24 hours prior to subjecting them to the SBR50 protocol with IL-6 treatment. The half-life of T40214 in tissues is ≥ 48 hours [25].

Nucleosome ELISA. Levels of histone-associated DNA fragments (nucleosomes) were determined in homogenates of snap-frozen liver using an ELISA method (Cell Death Detection ELISA[plus]; Roche Diagnostics, Manheim, Germany), as decribed [CITE TWO LIVER RAT T/HS PAPERS]. The nucleosome concentration for each liver sample was normalized for total protein concentration determined by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA). The final nucleosome concentration for each liver sample was the average of duplicate determinations.

Terminal deoxynucleotidyl transferase (TdT) mediated nick end labeling (TUNEL) staining. TUNEL staining to enzymatically detect the free 3'-OH termini was performed using the ApopTag Plus Peroxidase in situ Apoptosis Detection Kit from Chemicon International (now Millipore, Billerica, MA). Slides were rehydrated from xylene to PBS through a series of decreasing concentrations of ethanol and digested in proteinase K (20 ug/ml) for 3 minutes at 23°C. Endogenous peroxidases were quenched for 30 minutes in 3% hydrogen peroxide in PBS. TdT enzyme was diluted in TUNEL solution buffer then used as suggested by the manufacturer. Slides were counterstained with hematoxyllin. TUNEL positive cells were assessed microscopically by counting the total nuclei and the number of TUNEL-positive nuclei in twenty random 1000x fields by an experienced histologist, blinded to the treatment each rat received. Data is presented as the number of TUNEL positive cells per high power field (hpf).

RNA isolation and oligonucleotide microarray hybridization. Total RNA was isolated from 4-5 micron cryotome sections of liver using TRIzol® Reagent (Invitrogen,

Carlsbad, California) single step RNA isolation protocol followed by purification with RNeasy® Mini Kit (QIAGEN, Hilden, Germany) as instructed by the manufacturer. Gene expression profiling was performed with the Affymetrix Rat Array RAE 230A chips following Affymetrix protocols used within the Baylor College of Medicine Microarray Core Facility.

Microarray Analysis. We used GenespringGX (Agilent Technologies Inc, Santa Clara CA) software package for quality assessment, statistical analysis and annotation. Low-level analyses included background correction, quartile normalization and expression estimation using RMA-based analysis within Genespring. One-way analysis of variance (ANOVA) with contrasts was used for group comparisons on all genes and on the list of UPR entities. P-values were adjusted for multiple comparisons using the Benjamini-Hockberg method. The adjusted p-values represent false discovery rates (FDR) and are estimates of the proportion of "significant" genes that are false or spurious "discoveries". We used a FDR=5% as cut-off. The genechip used, RAE 230A, contained 15,923 probe sets representing 13,521 annotated genes or expressed sequence tags. A UPR gene entity list was created using both Ingenuity Pathway Analysis (IPA[®] Redwood City, CA) and the Gene Ontology Database[©], with keywords "endoplasmic reticulum stress, unfolded protein response". Three or more chips for each organ were hybridized using mRNA isolated from hearts and livers, respectively for each group comparison: Sham (4), T/HS-PBS (4) and T/HS-IL6 (4) and T/HS-IL6-GQ (3) groups.

Statistical Analysis. Statistical differences between experimental groups were

analyzed using one-way ANOVA and post-hoc analysis was performed using Student-

Newman-Keuls test.

RESULTS

T/HS-induced hepatocyte apoptosis is prevented by IL-6 resuscitation; the IL-6 effect is mediated, in part, by Stat3

To confirm our previous findings that T/HS induces liver apoptosis, we measured histone-associated DNA fragments (nucleosomes) in the livers of rats subjected to our T/HS protocol. Nucleosome levels were 13.1 times higher than sham ($p<0.001$, ANOVA; Table 1). The nucleosome results were confirmed by TUNEL staining (Table 1), which also demonstrated that hepatocytes represented the overwhelming majority of cells undergoing apoptosis (data not shown).

Nucleosome levels in the IL-6-resuscitated rats were decreased 3.3 times compared to those of the placebo group ($p<0.001$) and were similar to sham levels (Table 1). TUNEL staining confirmed these results (Table 1). The number of TUNEL-positive nuclei/hpf in the IL-6 group was decreased 14.2 times compared to the placebo group ($p<0.001$), to levels statistically similar to those of the sham group (Table 1).

Pretreatment of rats with the G-rich, quartet-forming oligonucleotide Stat3 inhibitor (T40214) was accompanied by a return of nucleosomes to levels similar to those of the placebo treated group and 11.2 fold higher that those of the IL-6 treated group ($p<0.001$; Table 1). Similarly, the number of TUNEL-positive nuclei/hpf in livers of rats from the T/HS-IL6-GQ group was 6 fold higher than that of the T/HS-IL6-treated group ($p<0.0001$); Table 1). Nucleosome levels and number of TUNEL-positive nuclei/hpf in

livers of rats pre-treated with a NS-ODN before T/HS and IL-6 resuscitation were indistinguishable from those of the IL-6 group (data not shown). Thus, pharmacological inhibition of Stat3 using T40214 in rats subjected to severe HS resuscitated with IL-6 completely blocked IL-6-mediated prevention of liver apoptosis.

Liver UPR transcriptome is significantly altered in T/HS

We investigated the impact of T/HS on the ER stress response at the transcriptome level, and then defined the role of this ER stress response on the observed reversible hepatic apoptosis.   Unbiased self-organizing clustering of our experimental animals into their intervention groups demonstrated the reproducible nature of the impact of T/HS on the UPR transcriptome (Figure 1).   Of the broad 185-gene UPR-associated entity list generated via literature review and Ingenuity Pathway Analysis (IPA®), 113 distinct gene entities were annotated and expressed across our chips after spot duplicates were removed. Using this list of 113 genes, 63 (56%) were significantly altered in one-way ANOVA ($p < 0.05$) among all three-group comparisons, T/HS vs. Sham, T/HS-IL6 vs. T/HS, and T/HS-IL6-GQ vs. T/HS-IL6. When the impact of T/HS was looked at specifically, 31 (27%) of those gene entities were significantly dysregulated when compared to sham, with 55% (17 of 31) significantly upregulated and 45% of gene transcripts downregulated. When asking the question of potential mediators of the protective effect of IL-6, 17 entities were significantly altered in both group comparisons. Taking known apoptotic function of these genes into context, we demonstrate that all UPR-associated genes with known potential pro-apoptotic function are upregulated following T/HS and subsequently normalized with IL-6 (Table 2). The most

dysregulated genes within this intergroup comparison were the chaperones, Heat Shock Protein 70 (25.6-fold), and Heat Shock Protein 40 (5.9-fold), the UPR transcription factor ATF4 (3.1-fold), and endoplasmic oxidoreductin-1-like protein (Ero1l) (9.8-fold) suggesting a strong impact on the protein folding mechanics both in the cytoplasm and the endoplasmic reticulum.

To assess which of these dysregulated genes may be impacted via IL-6 through Stat3, we incorporated animals pre-treated with a pharmacologic Stat3 inhibitor (GQ T40214) then resuscitated with IL-6 to animal resuscitated with IL-6 alone. Using this combined intergroup approach, we found 12 gene entities with significant dysregulation across all three-group comparisons (Table 3). Interestingly, we find that of the most dysregulated transcripts, the chaperones Hsp70 and Hsp40 demonstrate upregulation in T/HS. In animals in which hepatocyte apoptosis was prevented by receiving IL-6 at resuscitation, we find that Hsp70 and Hsp40 were further upregulated, suggesting a contribution to prevention of hepatocyte apoptosis. When Stat3 is pharmacologically inhibited, however, we find downregulation of these chaperones, suggesting IL-6 acts to upregulate Hsp70/40 via a Stat3-dependent mechanism not previously described.

T/HS-induced cardiomyocyte apoptosis is prevented by IL-6 resuscitation; the IL-6 effect is mediated, in part, by Stat3

To confirm our previous findings that T/HS induces cardiomyocyte apoptosis, we measured histone-associated DNA fragments (nucleosomes) in the hearts of rats subjected to our T/HS protocol. Nucleosome levels were increased 36 fold over sham in

T/HS rats (p<0.01, ANOVA; Table 1). The nucleosome results were confirmed by TUNEL staining (Table 1), which also demonstrated that cardiomyocytes represented the overwhelming majority of cells undergoing apoptosis (data not shown).

Nucleosome levels in hearts from IL-6 resuscitated rats were reduced by more than 7 fold compared to placebo treated rats undergoing T/HS (p<0.05, ANOVA). TUNEL assays of sections of rat hearts confirmed these findings with a similar 14-fold reduction (p<0.05, ANOVA; Table 1).

Pretreatment of rats with a Stat3 inhibitor was accompanied by a return of nucleosomes to levels similar to those of the placebo treated group. Nucleosome levels in the hearts of T/HS-IL6-GQ rats (Table 1) were increased 10-fold compared to hearts from IL-6 resuscitated rats (p<0.05, ANOVA; Table 1). Thus, pharmacological inhibition of Stat3 using T40214 in rats subjected to severe T/HS resuscitated with IL-6 completely blocked IL-6-mediated prevention of cardiomyocyte apoptosis.

Heart UPR transcriptome is significantly altered in T/HS

The results above demonstrate that cardiomyocyte apoptosis caused by T/HS is largely prevented with administration of IL-6 at time of resuscitation (Table 1). We investigated the impact of T/HS on the ER stress response at the transcriptome level, and then defined the role of this ER stress response on the observed reversible cardiomyocyte apoptosis. Using the previously described UPR gene entity list, we found that of the 113 genes present on the chip, 86 (76%) were significantly altered in one-way ANOVA

(p <0.05) among all three group comparisons, T/HS vs. Sham, T/HS-IL6 vs. T/HS, and

T/HS-IL6-GQ vs. T/HS-IL6. When the impact of T/HS was looked at specifically, 29

(26%) of those gene entities were significantly dysregulated when compared to sham,

with the majority, 79% (23 of 29) significantly upregulated and 6 gene transcripts

downregulated. When asking the question of potential mediators of the protective effect

of IL-6, 16 entities were significantly altered in both group comparisons (Table 4). The

direction of dysregulation induced by T/HS was reversed by IL-6 in all transcripts

identified. When taking known apoptotic functions of these genes and the impact of our

experimental model into context, we demonstrated that 4 of the 5 genes with known

pro-apoptotic function are upregulated following T/HS and subsequently normalized with

IL-6 (Table 4). The most dysregulated genes, those genes with > 2 fold change, within

this intergroup comparison were the chaperones, Hsp70 (10 fold), Hsp40 (3 fold), and

Hsp105 (2.5 fold), and the negative regulator of PERK, phosphoinositide-3-kinase

interacting protein 1 (Pik3ip1) (-3.0 fold), suggesting, as in the liver, a strong impact on

the protein folding mechanics both in the cytoplasm and the endoplasmic reticulum. In

contrast to the liver however, the heat shock protein chaperones, Hsp70 and Hsp40,

were downregulated in the hearts of IL-6-treated animals, indicating they likely are not

contributing to the apoptotic protection conferred by IL-6. When adding the comparison

of GQ T40214 to IL-6 group, we found 11 gene entities with significant dysregulation

across all three group comparisons, and, of those, 8 suggest potential IL-6 mediated

effect through Stat3 (Table 5).

DISCUSSION

Our findings provide the first-ever global description of the UPR transcriptome of the heart and liver following T/HS. We demonstrated that T/HS leads to significant cardiomyocyte and hepatocyte apoptosis, which is prevented through the Stat3-dependent actions of IL-6. We examined the UPR transcriptome to identify candidate gene transcripts responsible for T/HS-induced apoptosis. By utilizing an expanded repertoire of UPR members, both canonical and non-canonical, and the reproducible and measurable outcome of IL-6-preventable apoptosis in our model of T/HS, we were able to identify potential UPR modulators that significantly impact T/HS-induced cardiomyocyte and hepatocyte apoptosis.

In the liver, members of the heat shock family of protein folding chaperones, Hsp70 and Hsp40, emerged as significant potential non-canonical UPR modulators of hepatocyte apoptosis in our model of T/HS. This compares with findings in other models of organ injury, such as work done by Wang et al., which demonstrated that Hsp70 and its induction with geranylgeranylacetone (GGA) can protect against primary proximal tubule apoptosis and acute kidney damage in an ischemic injury model [26], and work done by Kuboki et al., which demonstrated in a partial liver I/R model that induction of Hsp70 with sodium arsenite reduced liver injury, as determined by transaminase levels and histology [27]. Besides their role in protein folding in the cytoplasmic space, heat shock proteins have been linked to the canonical UPR pathways of the endoplasmic reticulum. One example is Hsp72, a Hsp 70 family member, which has been shown to interact with the cytosolic domain of IRE1α, enhancing XBP1 splicing, and attenuating apoptosis *in vitro* [28]. Heat shock protein chaperones have also been shown to prevent

CHOP-induced apoptosis through the Hsp70-DnaJ chaperone pair inhibiting translocation of Bax to mitochondria *in vitro* [29].

Our findings are the first *in vivo* data linking the heat shock protein family to hepatocyte apoptosis via a Stat3-dependent mechanism in T/HS. Hsp70 and Hsp40 appear to contribute to an adaptive and protective process in the liver, demonstrating upregulation in T/HS and further upregulation in livers of IL-6-resuscitated animals, correlating with prevention of apoptosis. However, when animals were pretreated with a Stat3 inhibitor that blocked IL-6's prevention of apoptosis, these chaperone transcripts were downregulated. Thus, these findings suggest that IL-6, via a novel Stat3-dependent pathway, acts to superinduce Hsp70 and 40 transcripts in T/HS.

Interestingly, the canonical members of the UPR, while altered, were not the most dysregulated transcripts in the liver. CCAAT/-enhancer-binding protein homologous protein (CHOP), PERK, alpha subunit of eukaryotic initiation factor 2 (Eif2α), activating transcription factor 4 (ATF4), and calreticulin were significantly dysregulated (6 to -1.2 fold change) in T/HS (Supplemental Table 1). When considering those entities altered > 2-fold and taking into account known UPR and apoptotic functions of the canonical UPR members, only the transcriptional profile of ATF4 suggested a maladaptive contribution to hepatocyte apoptosis. However, this maladaptive role does not appear to be mediated through Stat3.

The heart demonstrated a distinctly different UPR transcriptional profile in comparison to the liver. When one considers the nature and functions of these organs, this is not unexpected. The liver is the largest glandular mass of tissue in the body and is highly secretory with both exocrine and endocrine function, whereas the heart, with myocyte predominance, is largely non-secretory with maintenance of biophysical function more paramount. The impact of T/HS on the canonical UPR transcriptome was even less in magnitude in the heart than in the liver. Significantly dysregulated canonical UPR transcripts included CHOP, PERK, X-box binding protein 1 (XBP1), Eif2α, and calreticulin (2.5 to -1.2 fold change) with only ATF4 dysregulated by more than 2 fold in response to T/HS (Supplemental Table 2). When taking into account known UPR and apoptotic function, CHOP, XBP1, and GADD34 exhibit transcriptional profiles suggestive of an adaptive role in T/HS-induced cardiomyocyte apoptosis. Given the fold-change was nominal (1.4 to 2-fold) however, further investigation is required to determine their true contribution to T/HS-induced apoptosis.

The protein folding chaperones, Hsp70 and Hsp40, which proved important modulators of apoptosis in the liver, were upregulated in the heart following T/HS, but were downregulated in animals in which IL-6 prevented cardiomyocyte apoptosis, suggesting these chaperones may play a maladaptive role in T/HS-induced cardiomyocyte apoptosis. The dichotomous nature of these chaperones' roles in the liver and heart in T/HS is supported by work in other models of organ injury. Indeed, previous studies have suggested that Hsp70 family proteins may serve to augment cardiac inflammation and contractile dysfunction [30, 31], and its downregulation in IL-6 treated animals would

support this hypothesis, as we have previously demonstrated that IL-6 acts to preserve

contractile function following T/HS [3].

In addition to providing a global description of the UPR transcriptome of the heart and

liver following T/HS, our findings demonstrate that IL-6, when utilized as a resuscitation

adjuvant, may augment a physiologic protective role of Hsp70 and Hsp40 via a novel

Stat3-dependent mechanism, thereby protecting against hepatocyte apoptosis. These

findings support the concept that modulators of Hsp70 or 40 may offer a novel

therapeutic strategy for prevention of apoptosis and ultimately hepatic dysfunction

following T/HS.

Table 1[§] Impact of T/HS without and with IL-6 on markers of apoptosis in the heart and liver

Intervention	Liver		Heart	
	Nucleosome[a]	**TUNEL**[b]	**Nucleosome**[a]	**TUNEL**[b]
Sham	$139 \pm 67^{*}$	$0.9 \pm 0.4^{**}$	$0^{\ddagger}$	$1.3 \pm 0.2^{\ddagger\ddagger}$
T/HS	$1874 \pm 127^{*\dagger}$	$27 \pm 3.6^{**,\dagger\dagger}$	$63 \pm 8^{\Delta,\ddagger}$	$16.2 \pm 2^{\Delta\Delta,\ddagger\ddagger}$
T/HS-IL6	$264 \pm 36^{\dagger\yen}$	$1.9 \pm 0.5^{\dagger\dagger,\yen\yen}$	$4 \pm 1^{\lozenge,\Delta}$	$8.5 \pm 0.2^{\lozenge\lozenge,\Delta\Delta}$
T/HS-IL6-GQ	$1556 \pm 241^{\yen}$	$12.3 \pm 1.1^{\yen\yen}$	$24 \pm 5^{\lozenge}$	$16.5 \pm 1^{\lozenge\lozenge}$

[§] $*,\dagger,\yen,**,\dagger\dagger,\yen\yen,\ddagger,\Delta,\lozenge,\ddagger\ddagger,\Delta\Delta,\lozenge\lozenge$ indicate group comparisons with statistical significance of $p<0.05$, one-way ANOVA. (a) Nucleosome data presented as mU/mg total protein. (b) TUNEL data presented as number of TUNEL-positive nuclei per high power field.

Table 2 Liver UPR Transcripts Significantly Altered in Both T/HS vs. Sham and T/HS/IL-6 vs. T/HS Comparisons

Gene Symbol	UPR Function	Apoptosis Function	Regulation		Fold Change	
			T/HS vs Sham	T/HS-IL6 vs T/HS	T/HS vs Sham	T/HS-IL6 vs T/HS
Hspa1a/Hspa1b (Hsp70)	Chaperone	Anti	up	up	25.6	5.5
Hspa1b (Hsp70-1b)	Chaperone	Anti	up	up	18.4	5.9
Ero1l	Disulfide Bond Formation	Anti	up	down	9.8	-3.8
Dnajb1 (Hsp40 Subunit b1)	Chaperone	Anti	up	up	5.9	2.7
Atf4	Transcription Factor	Anti/Pro	up	down	3.1	-2.0
Casp3 (caspase 3)	Apoptosis Signalling	Pro	up	down	1.8	-1.7
Eif2s1 (Eif2α)	Protein Translation	Anti	up	down	1.7	-1.6
Sels	Modulation of ATF6	Uknown	up	down	1.5	-1.3
Eif2ak3 (PERK)	UPR Sensory Molecule	Anti/Pro	up	down	1.4	-1.3
Psmb3	Proteasome Degradation	Anti	down	up	-1.2	1.3
Calr (calreticulin)	Chaperone	Anti	down	up	-1.2	1.2
Uba1	Ubiquitination	Anti	down	up	-1.3	1.2
Psme2	Proteasome Degradation	Anti	down	up	-1.3	1.4
Psme1	Proteasome Degradation	Anti	down	up	-1.3	1.2
Dyt1	ATPase	Anti	down	up	-1.4	1.4
Tmbim6 (Bax inhibitor 1)	Apoptosis Signalling	Anti	down	up	-1.5	1.3
Ccnd1	Cell Cycle Signalling	Anti	down	up	-3.4	2.3

Table 3 Liver UPR Transcripts Significantly Altered T/HS vs. Sham, T/HS/IL-6 vs. T/HS and T/HS/IL-6/GQ Comparisons

Gene Symbol	Regulation			Fold Change		
	T/HS vs Sham	T/HS-IL6 vs T/HS	T/HS-IL6-GQ vs T/HS-IL6	T/HS vs Sham	T/HS-IL6 vs T/HS	T/HS-IL6-GQ vs T/HS-IL6
Hspa1a/Hspa1b (Hsp70)	up	up	down	25.6	5.5	-9.0
Hspa1b (Hsp701b)	up	up	down	18.4	5.9	-10.8
Ero1l	up	down	down	9.8	-3.8	-1.3
Dnajb1 (Hsp40 subunit)	up	up	down	5.9	2.7	-3.4
Casp3 (Caspase 3)	up	down	up	1.8	-1.7	1.4
Sels	up	down	up	1.5	-1.3	1.4
Eif2ak3 (PERK)	up	down	down	1.4	-1.3	-1.3
Psmb3	down	up	up	-1.2	1.3	1.9
Uba1	down	up	up	-1.3	1.2	1.8
Psme2	down	up	up	-1.3	1.4	2.9
Psme1	down	up	up	-1.3	1.2	2.2
Tmbim6 (Bax inhibitor 1)	down	up	down	-1.5	1.3	-1.2

**Table 4** Heart UPR Transcripts Significantly Altered in Both T/HS vs. Sham and T/HS/IL-6 vs. T/HS Comparisons

Gene Symbol	UPR Function	Apoptosis Function	Regulation		Fold Change	
			T/HS vs Sham	IL6 vs T/HS	T/HS vs Sham	IL6 vs T/HS
Hspa1a/Hspa1b (Hsp70)	Chaperone	Anti	up	down	10	-6.4
Hspa1b (Hsp701b)	Chaperone	Anti	up	down	7.5	-4.9
Cebpb	Transcription factor	Pro	up	down	5.1	-1.7
Dnaja1 (Hsp40 subunit)	Co-chaperone	Anti	up	down	3.1	-2.3
Hsph1 (Hsp105)	Chaperone	Anti	up	down	2.5	-2.1
Dnajb1 (Hsp40 subunit)	Co-chaperone	Anti	up	down	2.2	-2
Nfe2l2	Transcription factor	Anti	up	down	2	-1.4
Ppp1r15a (GADD34)	Transcription factor	Pro	up	down	2	-1.4
Xbp1 (X-box-protein 1)	Transcription factor	Pro	up	down	1.5	-1.2
Tra1 (Hsp90b1)	Chaperone (ERAD)	Anti	up	down	1.4	-1.3
Calr (calreticulin)	Chaperone	Anti	up	down	1.4	-1.3
Ddit3 (CHOP)	Transcription factor	Pro	up	down	1.4	-1.3
Serp1	protects unfolded proteins from ERAD	Anti	up	down	1.3	-1.2
Sp1	Transcription factor	Pro	down	up	-1.2	1.3
Sels	modulates ATF6	Unknown	down	up	-1.5	1.3
Pik3ip1	negative regulator of PERK	Anti	down	up	-3	1.4

Abbreviation: ERAD, endoplasmic reticulum-associated degradation.

Table 5 Heart UPR Transcripts Significantly Altered Across T/HS vs. Sham, T/HS/IL-6 vs. T/HS, and T/HS/IL-6/GQ vs.

T/HS/IL-6 Comparisons

Gene Symbol	Regulation			Fold Change		
	T/HS vs Sham	T/HS-IL6 vs T/HS	T/HS-IL6-GQ vs T/HS-IL6	T/HS vs Sham	T/HS-IL6 vs T/HS	T/HS-IL6-GQ vs T/HS-IL6
Dnaja1 (Hsp40 subunit)	up	down	up	3.1	-2.3	2.0
Hsph1 (Hsp105)	up	down	up	2.5	-2.1	1.5
Nfe2l2	up	down	up	2.0	-1.4	2.0
Ppp1r15a (GADD34)	up	down	up	2.0	-1.4	1.4
Xbp1 (X-box-protein 1)	up	down	up	1.5	-1.2	1.8
Tra1	up	down	up	1.4	-1.3	1.2
Calr (calreticulin)	up	down	up	1.4	-1.3	1.4
Ddit3 (CHOP)	up	down	up	1.4	-1.3	5.5
Sp1	down	up	up	-1.2	1.3	1.6
Sels	down	up	up	-1.5	1.3	3.1
Pik3ip1	down	up	up	-3.0	1.4	1.8

Figure 1
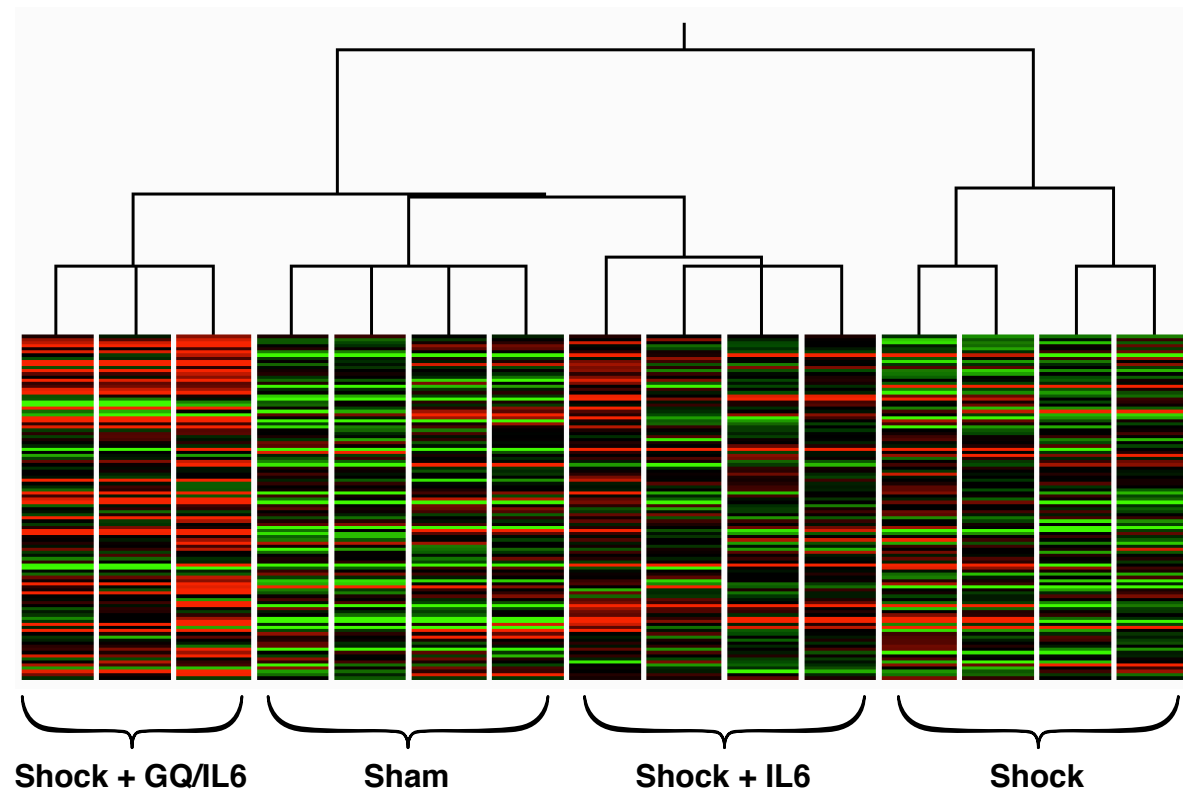
Titles and Legends to Figures

Figure 1

Unbiased self-organizing map of experimental animals confined to 113 UPR-associated gene entities on whole liver preparations. Clustering performed using Pearson Absolute similarity measure, expression data normalized to chip standards for clustering.

Supplemental Table 1 Liver UPR Transcripts Significantly Altered T/HS vs. Sham

Gene Symbol	T/HS vs. Sham	
	Regulation	Fold change
Hspa1a/Hspa1b	up	25.6
Hspa1b	up	18.4
Ero1l	up	9.8
Hsp90aa1	up	6.6
Ddit3	up	6.1
Dnajb1	up	5.9
Hsph1	up	5.9
Ppp1r15a	up	3.6
Atf4	up	3.1
Dnaja1	up	2.4
Nfe2l2	up	2.2
Hspe1	up	2.0
Casp3	up	1.8
Eif2s1	up	1.7
Hsp90ab1	up	1.6
Sels	up	1.5
Eif2ak3	up	1.4
Ins2	down	-1.2
Ube4b	down	-1.2
Psmb3	down	-1.2
Calr	down	-1.2
Tnf	down	-1.2
Vcp	down	-1.2
Uba1	down	-1.3
Psme2	down	-1.3
Psme1	down	-1.3
Dyt1	down	-1.4
Tmbim6	down	-1.5
Gata4	down	-1.6
Herpud1	down	-1.8
Ccnd1	down	-3.4

Supplemental Table 2 Heart UPR Transcripts Significantly Altered T/HS vs. Sham

Gene Symbol	T/HS vs. Sham	
	Regulation	Fold change
Hspa1a/Hspa1b (Hsp70)	up	10.0
Hspa1b (Hsp70 subunit)	up	7.5
Cebpb	up	5.1
Dnaja1 (Hsp40 subunit)	up	3.1
Atf4	up	2.5
Hsph1 (Hsp105)	up	2.5
Dnajb1 (Hsp40 subunit)	up	2.2
Nfe2l2	up	2.0
Ppp1r15a	up	2.0
Hsp90aa1	up	2.0
Xbp1 (Xbox-1)	up	1.5
Hspa5	up	1.4
Tra1 (Hsp90b1)	up	1.4
Calr (calreticulin)	up	1.4
Ddit3 (CHOP)	up	1.4
Bag1 (BCL2-associated athanogene)	up	1.3
Serp1	up	1.3
Eif2s1 (Eif2α)	up	1.3
Aars	up	1.3
Psmb4 (proteosome subunit)	up	1.2
Ero1l	up	1.2
Hspb7 (Cardiovascular heat shock protein)	up	1.2
Psma2	up	1.2
Tnf (tumor necrosis factor α)	down	-1.1
Sp3	down	-1.1
Eif2ak3 (PERK)	down	-1.2
Sp1	down	-1.2
Sels	down	-1.5
Pik3ip1	down	-3.0

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Title:
Protect the cell to protect the host, linking the unfolded protein response to alveolar epithelial cell apoptosis and susceptibility to *Pseudomonas aeruginosa* pneumonia in the injured host.

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Background:
   Trauma is the most common cause of death in the US in those under the age of 45. Ventilator-associated pneumonia (VAP), contributes to significant mortality in those surviving initial trauma. Acute lung injury (ALI) seen in trauma is an important risk factor for VAP. *Pseudomonas aeruginosa* (PA) is a common etiology of VAP, carrying high mortality due, in part, to antimicrobial resistance. Thus, there is need for novel therapeutic strategies such as modulating the susceptibility of the injured host to pneumonia.
   Respiratory epithelium is essential to innate immunity against PA pneumonia, and injury to this epithelium is a hallmark of ALI. Type II alveolar epithelial cells (AEC II) produce surfactant protein D (SPD), an innate immune protein contributing to clearance of PA. We have previously shown in a rodent model of trauma/hemorrhagic shock (T/HS) that apoptosis peaks 1 hr following resuscitation, 82% of apoptotic cells are AECs, SPD is decreased, and IL6 given at resuscitation prevents these abnormalities. AEC IIs are the major secretory cells of the alveolar space. In other models of ischemia/reperfusion injury involving secretory cells, overwhelming endoplasmic reticulum (ER) stress can lead to apoptosis via the unfolded protein response (UPR). Here, we describe the potential contribution the UPR to AEC apoptosis, and innate immune dysfunction, following T/HS using SPD as a marker.

Results:
SPD is reduced 1.8 fold in animals undergoing T/HS and is restored with IL6, compared to sham. T/HS confers an 80% increased mortality and 10 fold bacterial burden increase compared to sham in PA pneumonia. The chaperone, heat shock protein 70, is a potential adaptive non-canonical UPR component that protects against AEC-II apoptosis, and UPR sensory molecules, ATF4 and IRE1α, are potential maladaptive mediators of ER stress contributing to AEC II apoptosis following T/HS.

Methods:
Transcriptome analysis was performed on sham, T/HS, T/HS with IL6 animal lungs using microarray containing 113 genes in the UPR transcriptome. Lung bacterial burden and mortality performed in a 2 hit model of T/HS followed by PA pneumonia.

Conclusion:
These findings provide a potential novel mechanism for therapeutic intervention against ALI and VAP following trauma through modulation of the UPR.

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Title: Contribution of Heat Shock Proteins 70 and 40 to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock

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Background: Given mounting antimicrobial resistance and few anti-pseudomonals in development, there is a need for novel strategies to prevent ventilator-associated pneumonia (VAP) caused by Pseudomonas aeruginosa (PA), a common cause of death following trauma complicated by hemorrhagic shock (T/HS). We previously demonstrated in a rat model of T/HS that type I and II alveolar epithelial cells (AEC) undergo apoptosis, which is accompanied by a 50% reduction in surfactant protein-D (SPD). When intratracheal installation of PA followed T/HS, there was a 10-fold increase in PA bacterial burden and PA pneumonia mortality increased 80%. Notably, T/HS-mediated AEC apoptosis, SPD reduction, and PA pneumonia susceptibility were prevented by IL-6 via a Stat3-dependent pathway. The basis for T/HS-induced impaired innate immunity and its prevention by IL-6-activated Stat3 is unknown. The unfolded protein response (UPR) alleviates endoplasmic reticulum (ER) stress in secretory cells like AEC II when the stress is mild-to-moderate, but has recently been shown to cause apoptosis when ER stress is severe.

Objective: Determine the UPR's contribution T/HS-mediated AEC apoptosis and its prevention by IL-6.

Design/Methods: Global UPR transcriptome analysis was performed using Affymetrix chips of 4 groups of rats: sham, T/HS, T/HS+IL-6, and T/HS+IL-6 pretreated with a Stat3 inhibitor. Using Ingenuity Pathway Analysis© and GO database, we generated a list of 185 UPR-associated genes; 113 were annotated on the chip. Genespring© was used to interpret the impact of T/HS and interventions on those mediators of the UPR.

Results: T/HS altered levels of 30% of UPR transcripts compared to sham ($p < 0.05$, ANOVA). All significantly dysregulated pro-apoptotic UPR transcripts normalized with IL-6. The protein folding chaperones, heat shock proteins 70 and 40 (Hsp70, Hsp40) were identified as the most dysregulated UPR mediators in T/HS (>25 fold and >9 fold, respectively; $p < 0.05$, ANOVA). Hsp70 and Hsp40 were further upregulated >5 fold in T/HS+IL-6 animals and downregulated >9 fold in T/HS+IL-6 animals with Stat3 inhibition ($p < 0.05$, ANOVA).

Conclusions: Thus, Hsp70 and Hsp40 play an adaptive role in T/HS-mediated AEC apoptosis that is augmented by IL-6 through a novel Stat3-dependent pathway. Increasing these chaperones within the lungs using clinically available proteostasis modulators may prevent AEC apoptosis and PA VAP following T/HS.

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Title: Contribution of Heat Shock Proteins 70 and 40 to Prevention of Alveolar Epithelial Cell Apoptosis in Trauma Complicated by Hemorrhagic Shock

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Given mounting antimicrobial resistance and few anti-pseudomonals in development, there is a need for novel strategies to prevent ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* (PA), a common cause of death following trauma complicated by hemorrhagic shock (T/HS). We previously demonstrated in a rat model of T/HS that type I and II alveolar epithelial cells (AEC) undergo apoptosis, which is accompanied by a 50% reduction in surfactant protein-D (SPD). When intratracheal instillation of PA followed T/HS, PA bacterial burden increased 10 fold and PA pneumonia mortality increased 80%. T/HS-mediated AEC apoptosis, SPD reduction, and PA pneumonia susceptibility were prevented by IL-6 via a Stat3-dependent pathway. The basis for T/HS-induced impaired innate immunity and its prevention by IL-6-activated Stat3 is unknown. The unfolded protein response (UPR) alleviates endoplasmic reticulum (ER) stress in secretory cells like AEC II and has recently been shown to cause apoptosis when ER stress is severe. We sought to define the UPR's contribution T/HS-mediated AEC apoptosis and its prevention by IL-6.

Global UPR transcriptome analysis was performed using Affymetrix chips of 4 groups of rats: sham, T/HS, T/HS+IL-6, and T/HS+IL-6 pretreated with a Stat3 inhibitor. Via Ingenuity Pathway Analysis and GO database, we generated a list of 185 UPR-associated genes; 113 were annotated on the chip. Genespring was used to interpret the impact of T/HS and interventions on those mediators of the UPR.

T/HS altered levels of 30% of UPR transcripts compared to sham ($p < 0.05$, ANOVA). All dysregulated pro-apoptotic UPR transcripts normalized with IL-6. The protein folding chaperones, Hsp70 and Hsp40 were identified as the most dysregulated UPR mediators in T/HS (>25 fold and >9 fold, respectively; $p < 0.05$, ANOVA). Hsp70 and Hsp40 were further upregulated >5 fold in T/HS+IL-6 animals and downregulated >9 fold in T/HS+IL-6 animals with Stat3 inhibition ($p < 0.05$, ANOVA).

Thus, Hsp70 and Hsp40 play an adaptive role in T/HS-mediated AEC apoptosis that is augmented by IL-6 through a novel Stat3-dependent pathway. Increasing these chaperones within the lungs using available proteostasis modulators may prevent AEC apoptosis and PA VAP following T/HS.

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